

# THE ONCOGENIC PROPERTIES OF AMOT80 IN MAMMARY EPITHELIA

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## **DEDICATION**

I dedicate this dissertation to my wife Marcia R. Ranahan, and to my parents, William and Lynda Ranahan.

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## **ABSTRACT**

William P. Ranahan

### **THE ONCOGENIC PROPERTIES OF AMOT80 IN MAMMARY EPITHELIA**

While breast cancer is the second most commonly diagnosed cancer worldwide, its causes and natural history are not well defined. The female mammary organ is unique in that it does not reach full maturity until the lactation cycle following pregnancy. This cycle entails extensive growth and reorganization of the primitive epithelial ductal network. Following lactation, these same epithelial cells undergo an equally extensive program of apoptosis and involution. The mammary gland's sensitivity to pro-growth and pro-apoptotic signals may partly explain its proclivity to develop cancers.

For epithelial cells to become transformed they must lose intracellular organization known as polarity as differentiated epithelial tissues are refractory to aberrant growth. One essential component of epithelial to mesenchymal transition is the intrinsic capacity of cells to repurpose polarity constituents to promote growth. Recently, a novel mechanism of organ size control has been shown to repurpose the apical junctional associated protein Yap into the nucleus where it functions as a transcriptional coactivator promoting growth and dedifferentiation.

The focus of my work has been on a family of adaptor proteins termed Amots that have been shown to scaffold Yap and inhibit growth signaling. Specifically, I have shown that the 80KDa form of Amot, termed Amot80, acts as a dominant negative to the other Amot proteins to promote cell growth while reducing cell differentiation. Amot80 was found to promote the prolonged activation of MAPK signaling. Further, Amot80 expression was also found to enhance the transcriptional activity of Yap. This effect likely underlies the ability of Amot80 to drive disorganized overgrowth of MCF10A cells grown in Matrigel<sup>TM</sup>. Overall, these data suggest a

mechanism whereby the balance of Amot proteins controls the equilibrium between growth and differentiation within mammary epithelial tissues.

Clark D. Wells, PhD, Chair

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## LIST OF ABBREVIATIONS

|         |  |
|---------|--|
| ACCH    | Amot coiled-coil homology                                      |
| AJ      | Adherens junctions   |
| aPKC    | Atypical Protein Kinase C                                      |
| Amot    | Angiomotin   |
| Amot80  | 80KDa Amot protein product                                     |
| Amot130 | 130KDa Amot protein product                                    |
| ATCC    | American Type Culture Collection                               |
| ATM     | Ataxia telangiectasia mutated                                  |
| ATR     | Ataxia telangiectasia and Rad-3-related                        |
| BCA     | Bicinchoninic acid   |
| BTIC    | Breast cancer tumor initiating cell                            |
| CDC42   | Cell Division Control protein 42                               |
| cDNA    | Complimentary DNA  |
| CFP     | Cerulean fluorescent protein                                   |
| CPM     | Counts per minute  |
| Crb     | Crumb  |
| CTGF    | Connective tissue growth factor                                |
| Cyr61   | Cysteine-rich angiogenic inducer 61                            |
| DAPI    | 4', 6 diamidino 2 phenylindole                                 |
| DCIS    | Ductal carcinoma in situ                                       |
| DEPC    | Diethylpyrocarbonate   |
| DMEM    | Dulbecco's Modified Eagle Medium                               |
| E2      | Estradiol  |
| EGF     | Epidermal growth factor  |
| EGFR    | Epidermal growth factor receptor                               |
| Elk1    | Ets-like gene 1  |
| EMT     | Epithelial to mesenchymal transition                           |
| ER      | Estrogen receptor  |
| ERK     | Extracellular signal-regulated kinase                          |
| FBS     | Fetal bovine serum   |
| GEF     | Guanine nucleotide exchange factor                             |
| GFP     | Green fluorescent protein                                      |
| GH      | Growth hormone   |
| Grb2    | Growth factor receptor-bound protein 2                         |
| GTP     | Guanosine triphosphate   |
| Her2    | Human Epidermal Growth Factor Receptor 2                       |
| IDC     | Invasive ductal carcinoma                                      |
| INT     | Tetrazolium salt   |
| JAM     | Junctional adhesion molecule                                   |
| Lats    | Large tumor suppressor   |
| LCIS    | Lobular carcinoma in situ                                      |
| LDH     | Lactate dehydrogenase  |
| IrECM   | Laminin-rich extracellular matrix                              |
| MAPK    | Mitogen-activated protein kinase                               |
| MEK     | MAPK/ERK kinase  |
| Mst     | Mammalian STE20-like protein kinase                            |
| NEDD    | Neural-precursor-cell-expressed developmentally down-regulated |
| NFB-A   | Nuclear Fractionation Buffer A                                 |

|          |   |
|----------|---|
| PALS1    | Protein associated with Lin Seven 1                                 |
| PAR3     | Partitioning defective 3  |
| PAR6     | Partitioning defective 6  |
| PATJ     | PALS1-associated tight junction protein                             |
| PBS      | Phosphate buffered saline   |
| PCNA     | Proliferating cell nuclear antigen                                  |
| PDZ      | Post-synaptic density, Discs large, Zonula occludens                |
| PEI      | Polyethylenimine  |
| PLC      | Pregnancy lactation cycle   |
| PM       | Plasma membrane   |
| PR       | Progesterone receptor   |
| PTEN     | Phosphatase and tensin homolog                                      |
| qRT-PCR  | Quantitative real time polymerase chain reaction                    |
| Rac1     | Ras-related C3 Botulinum toxin substrate 1                          |
| Ras      | Rat sarcoma   |
| RIPA     | Radioimmunoprecipitation assay buffer                               |
| RT       | Reverse transcriptase   |
| RTK      | Receptor tyrosine kinase  |
| SDS-PAGE | Sodium-dodecyl sulfate polyacrylamide gel electrophoresis           |
| SH2      | Src Homology 2  |
| SOS      | Son of sevenless  |
| SRF      | Serum response factor   |
| STAT     | Signal transducers and activators of transcription                  |
| Taz      | Transcriptional co-activator with PDZ-binding motif TEAD Tea-domain |
| TEAD     | TEA-domain containing   |
| TGF      | Transforming growth factor  |
| TJ       | Tight junction  |
| Yap      | Yes-associated protein  |
| ZO1      | Zona occludens 1  |
| ZONAB    | ZO-1-associated nucleic acid binding protein                        |

## **CHAPTER 1. INTRODUCTION**

## 1.1 Breast Cancer Epidemiology and Causal Factors

Breast cancer is the second most common cancer worldwide (Ferlay et al., 2010). It is the most frequently diagnosed cancer in women living in the United States and Europe (Ferlay et al., 2007), and is the second leading cause of cancer deaths in American women. The American Cancer Society estimates that 232,000 new cases will be diagnosed in 2013 with close to 40,000 deaths. Approximately 1 in 8 women in the United States will develop invasive breast cancer during their lifetime. Incidence of breast cancer diagnosis has decreased in recent years which would suggest advancements in treatment efficacy; however, the largest decline in the past decade was a 7% decrease in 2003. This decrease was attributed to a decline in post-menopausal hormone therapy. During the 1980s and 1990s incidents of breast cancer rose steadily until the link was established between the use of hormone therapy and increased risk for breast cancer and heart disease (Rossouw, 2002). This link is suggestive of a causative role for hormone induced aberrant cell growth in the breast. The recent decreases in breast cancer incidence, while encouraging, are not due to actual advancements in treatment. Early detection remains the best preventative measure since the causal factors involved in breast cancer development remain unclear. An important research focus is clarifying the relationship between extracellular cues, such as hormones and growth factors, and regulation of growth and de-differentiation.

The term “breast cancer” refers to a collection of breast diseases which present with diverse histopathological, genetic, and molecular profiles. Breast cancers are classified primarily by location of origin, and secondarily by apparent invasive capacity. The most common type of non-invasive breast cancer is ductal carcinoma *in situ* (DCIS) which is also referred to as intraductal carcinoma. This cancer is characterized by non-invasive cells proliferating within the lumen of a duct. DCIS can be detected by mammogram, and if diagnosed early, is very likely to have a successful treatment outcome. Interestingly, aberrant growth of non-invasive cells within the lumen of the mammary lobe, termed lobular carcinoma *in situ* (LCIS), is not considered a true

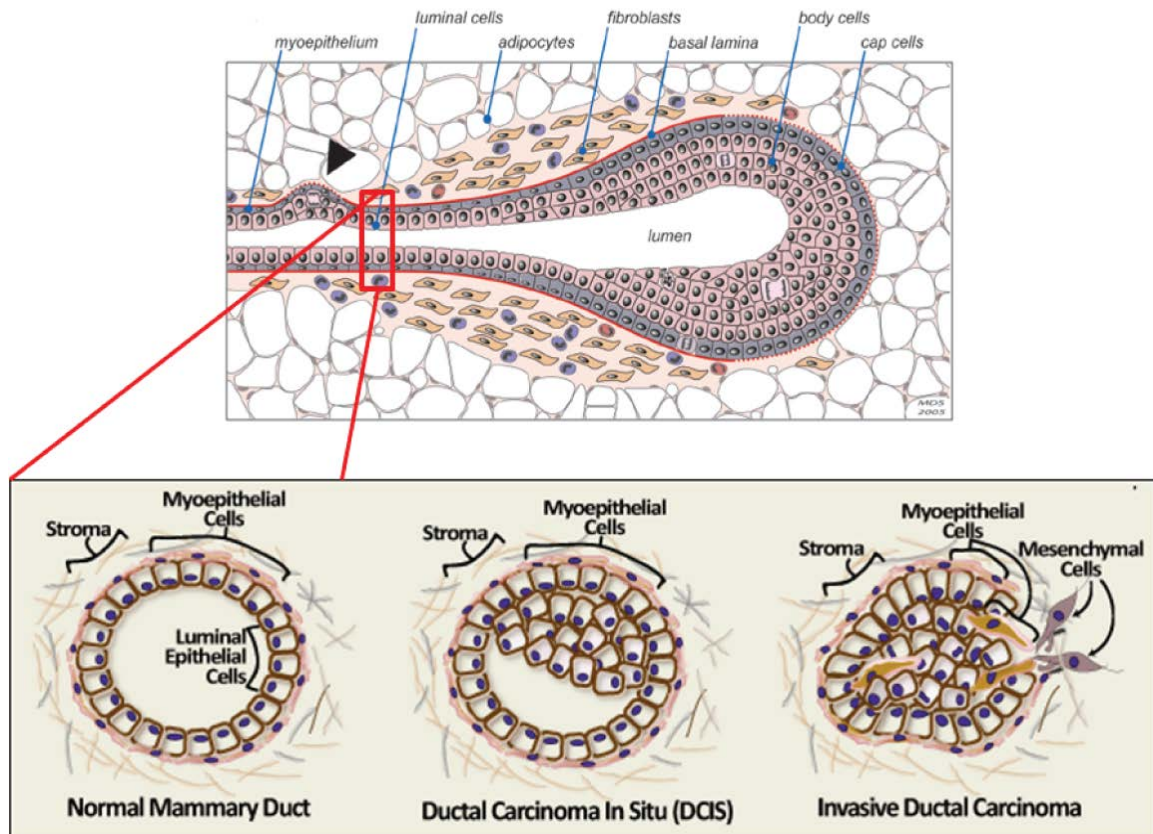
cancer or even a pre-cancer. Invasive ductal carcinoma (IDC) is the most common type of breast cancer. This cancer is characterized by the presence of cells both within a milk duct and infiltrating through the ductal lining into the adipose tissue and surrounding stroma. At this stage of the disease cancer cells may migrate into the vasculature and metastasize throughout the body (Figure 1-1).

To date, the best understood factors which contribute to breast cancer development are gender, age, and genetics. Women are about 100 times more likely to develop breast cancer than men. Whereas 2 out of 3 cases of invasive breast cancer are diagnosed in women over the age of 55, only 1 in 8 cases occur in women younger than 45 (Rossouw, 2002). Interestingly, only 5% to 10% of breast cancers are thought to be directly attributable to inherited genetic mutations (Rossouw, 2002). The most common inherited genetic mutation occurs within the BRCA1 and BRCA2 genes which can increase the likelihood of presenting with breast cancer by up to 80% in patients with at least one mutation (Kelsey, 1979). Genes involved in DNA damage repair, such as ataxia telangiectasia mutated (ATM) (Ahmed and Rahman, 2006), pro-apoptotic genes such as TP53 (Gasco et al., 2002), and protein phosphatases like phosphatase and tensin homolog (PTEN) (Zhang and Yu, 2010), have all been implicated in breast cancer development. Given the heterogeneity of this disease it is not surprising that no one factor has emerged as *the* dominant cause of breast cancer progression.

Since breast cancer has few known causal factors, only a handful of effective therapeutic targets are currently available (Key et al., 2001). Further confounding treatment is the diversity within the genetic and molecular makeup of this disease. Current hormonal therapies provide immediate reduction of tumor mass but may result in promotion of hormone resistant cancer cells (Perou et al., 2000). These resistant tumors often display increased invasive capacity and poor patient outcome (Hudis and Gianni, 2011).

Given the prevalence of breast cancer it may be stated that the female mammary organ is more likely than any other organ to generate tumors. This observation leads to the question of why this organ is so unusually susceptible to aberrant growth. Interestingly, the mammary organ is unlike most organs in the body. To further clarify why female mammary epithelial cells are among the highest generators of cancer cells an analysis of mammary organogenesis is appropriate.





**Figure 1-1 Breast Cancer Progression Illustration.**

Epithelial cells lining the mammary ducts escape contact mediated inhibition of cell growth and begin to proliferate into the lumen of the duct. Intrinsic and extrinsic factors such as loss of apical-basal polarity and increased sensitivity to growth factors contribute to aberrant cell growth and luminal filling until cells break through the epithelial barrier. Epithelial cells which have lost the asymmetrical distribution of growth factor receptors and have initiated pro-growth signaling pathways fully transition into a mesenchymal phenotype capable of metastasis. Figure adapted from (Sternlicht, 2006).

## 1.2 Mammary Organogenesis and Molecular Mechanisms Regulating Breast Development

Mammary organogenesis is unique among the developmental systems of the body. Unlike organs of the body which reach a relatively mature state during embryonic life, the mammary glands do not reach full maturity until the pregnancy lactation cycle (PLC) (Russo and Russo, 2004). The critical stages of mammary development include fetal and infant growth, pubertal expansion, pregnancy and lactation-associated remodeling, and post-lactational and post-menopausal involution (Figure 1-2) (Hassiotou and Geddes, 2013).

During embryonic life the mammary crest and primitive mammary buds form. Infancy and pre-puberty are characterized by minimal growth as the mammary glands remain in an immature resting state with virtually no differences in structure between males and females (Russo and Russo, 2004).

In the pubertal female, profound hormonal changes result in ovulation and the establishment of regular menstrual cycles. These developments induce rapid breast growth, due mainly to the deposition of adipose tissue within the mammary gland. During this stage both mammary epithelial cells and their surrounding stroma undergo dramatic changes regulated by an ovarian hormonal circuit acting on mammary stem cells located in the basal ductal layer (Russo J, 1992). These changes include elongation and branching of the existing ducts into secondary ducts terminating at bi-layered epithelial buds termed lobules (Neville MC, 2002).

Mammary organogenesis culminates during the PLC which involves a complete remodeling of the breast ducts, resulting in a functional milk secreting organ. Ductal branching, alveolar morphogenesis, and secretory differentiation are tightly regulated by the lactogenic hormone complex. The lactogenic hormone complex includes estrogen, progesterone, and prolactin. Levels of these three hormones change dynamically throughout the PLC and have established roles in mediating growth, differentiation, and involution (Pang and Hartmann, 2007). More recently, several additional growth factors and hormones have been identified as key

regulators of ductal branching and morphogenesis including epidermal growth factor (EGF), transforming growth factor alpha and beta (TGF $\alpha$  and TGF $\beta$  respectively), and growth hormone (GH) (Mark, 2006). The complexity of such a wide range of growth factors and hormones continues to be a barrier to elucidating their normal functions within the mammary gland. What role these growth factor pathways play during tumorigenesis remains poorly demonstrated. In contrast, the roles of the lactogenic hormones are better understood.

During the period of active breast feeding, blood levels of prolactin remain high and are associated both with milk synthesis and cell proliferation (Neville MC, 2002). Prolactin is therefore implicated in maintenance of secretory cells. During the weaning stage, milk production is reduced. PLC hormones and growth factor signaling gradually decreases resulting in slower rates of cell growth and metabolism (Hassiotou and Geddes, 2013). Although there are many inputs involved in signaling to the epithelial cells in the mammary gland during this period, the net effect during the weaning stage is a coordinated regression of lactation. This process includes both decreases in the number of secretory cells as well as decreased metabolic rates within the secretory cells which remain active (Stein et al., 2007).

The final step in the PLC remains the least well understood (Stein et al., 2007). Within the span of a few months the mammary gland switches from a robust growth and remodeling phase to an equally robust program of apoptosis and whole organ involution. Recent studies have shed light on the relevant signaling cascades involved in this fine-tuned process (Clarkson et al., 2004; Radisky and Hartmann, 2009; Stein et al., 2004). The process of involution begins with a high degree of epithelial cell death, continues with lobular and ductal clearing of cellular debris, and finishes after redevelopment of mammary adipose tissue and stromal remodeling (Stein et al., 2007). During this period of involution the mammary gland not only decreases in size, but is completely reorganized. Once apoptotic debris are cleared, many ductal networks are completely lost while remaining networks form new connections (Stein et al., 2007). Involution is triggered largely by the levels of lactogenic hormones, but not exclusively so. In addition to hormonal

signaling, localized stromal cells including macrophages and eosinophils play particularly important roles throughout the PLC (Gouon-Evans et al., 2002). Remarkably, the pregnancy lactation cycle can occur multiple times over the reproductive life of a female until a final stage of involution is passed concurrent with menopause.

It is noteworthy that completion of at least one PLC confers long term protection against the development of breast cancer (Key et al., 2001). This is thought to be due to the down regulation of cancer related pathways during the PLC. Analysis of genes expressed in the mammary gland during the PLC vs. puberty to pregnancy reveals a down regulation of genes involved in various cancers (Zhao et al., 2012b). Genes involved in mediation of cell-cell contacts, such as the tight junctions, are among the highest classes upregulated during puberty to pregnancy. This same class of genes is also among the highest down regulated during the PLC (Zhao et al., 2012b). These data suggest that polarity proteins involved in regulation of cell-cell contacts, if aberrantly expressed, may cause cancer.

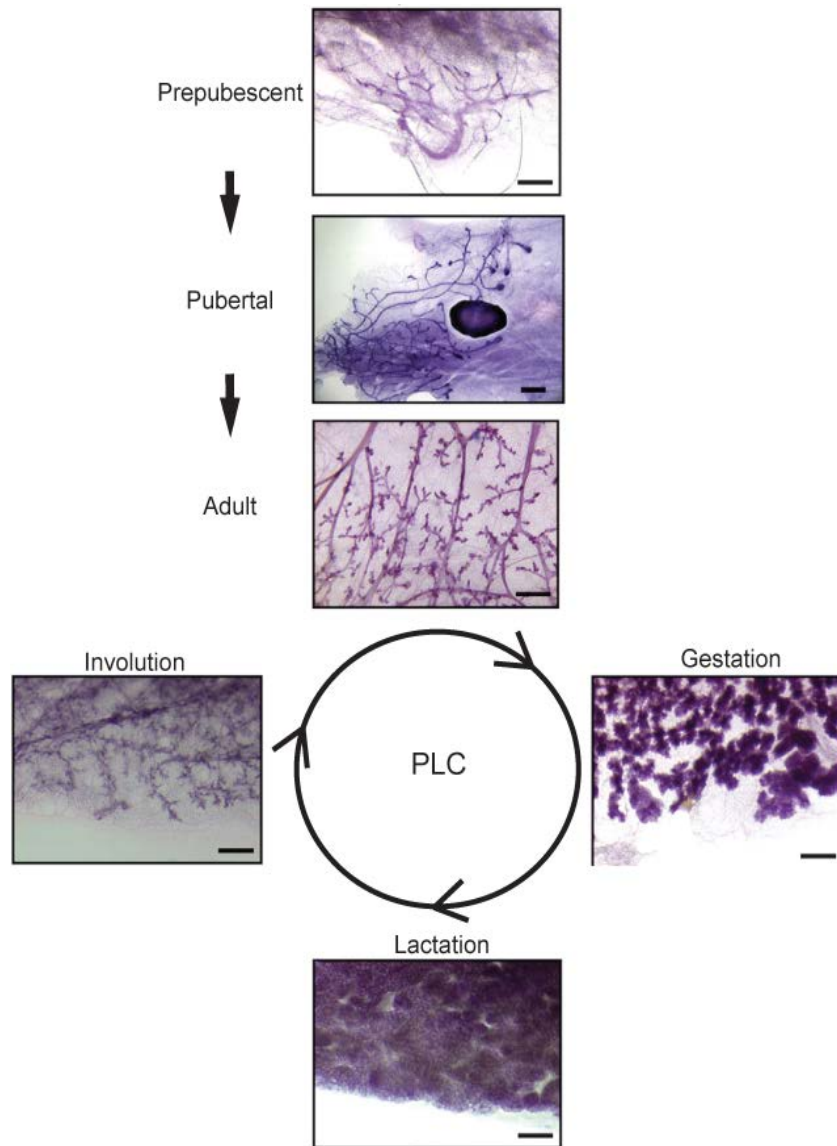
Recent work has demonstrated that epithelial cells within the lumen of terminal end buds transform into a mesenchymal state during the PLC (May et al., 2011). Epithelial to mesenchymal transition (EMT) is recognized as a hallmark of increased malignancy (Hanahan and Weinberg, 2000). The ability of epithelial cells within the lumen of terminal end buds to undergo EMT further suggests that at least some of the pathways involved in normal mammary organogenesis may be aberrantly activated during cancer progression.

Development and redevelopment of female breasts requires a wide range of hormones and growth factors. During the course of development the mammary gland undergoes both dramatic growth as well as apoptotic programs. Each menstrual cycle compounds epithelial development, at the level of organ structure as well as cellular responsiveness to differentiation (Russo J, 1992; Russo and Russo, 2004). The mammary organ is therefore unique in its ability to grow and initiate secretory functions as needed. The ability to invade, proliferate,

differentiate, and apoptose, comes with an intrinsic sensitivity to growth and de-differentiation.

This sensitivity may partly explain why breast cancer is so prevalent.

Surprisingly, although the mammary gland as a whole undergoes massive reorganizations, as described above, the location of tumor formation in the breast is not random. The vast majority of breast tumors originate from epithelial cells that line ducts. In order to understand why these cells in particular generate the vast majority of breast tumors it is necessary to consider both the environmental influences and the internal growth signaling pathways involved in maintaining steady-state physiological processes. The epithelial cells lining the secretory ducts are part of a larger network of cells and tissues. Therefore, clear understanding of the microenvironment surrounding ductal epithelia is necessary to understand the origin of breast cancer.



**Figure 1-2 Mammary Organogenesis**

Mammary gland development involves a linear phase which is terminated by pregnancy, beginning the pregnancy-lactation cycle (PLC). This cycle can be repeated throughout the reproductive life span of the female and ends with a period of involution during menopause. A series of murine mammary whole mounts illustrating distinct mammary morphology during the indicated stages of development from embryonic through to adult, postnatal prepubescent mammary gland (2-wk old), rapidly elongating pubertal mammary gland with bulbous TEBs (5-wk old), and the fully developed adult gland (10-wk old). The major reproductive phases that involve extensive remodelling are gestation (day 18.5 gestation), which shows extensive lobuloalveolar differentiation; lactation, which highlights a functionally differentiated gland; postlactational involution (day 8 post lactation), which shows the loss of secretory epithelium. Size bar = 1 mm (Prepubertal, Pubertal and Adult); 50  $\mu$ m in all other panels. Figure adapted from (Khokha and Werb, 2011).

### 1.3 Organization of the Mammary Organ and Origins of Breast Cancer

Mammary organogenesis is a life-long process of dynamic reciprocity between the many cell types within the breast and their microenvironment (Xu et al., 2009). It is therefore important to maintain a holistic view of this organ while focusing on the immediately relevant cell types and their key effectors of growth and differentiation. The breast is composed of both secretory and fatty tissue which is supported by a network of fibrous connective tissue (Figure 1-3). Storage and transport of milk is mediated by a ductal network connecting the glandular secretory tissue to the nipple (Hassiotou and Geddes, 2013). The glandular tissue is composed of alveoli, 10-100 comprise a lobule, and several lobules comprise a lobe (Hartmann, 2007). Each lobe of the breast is considered a closed unit (Going and Moffat, 2004). The post-pubertal, non-lactating, breast consists of ductal epithelial cells embedded in a fibrous stroma. The wall of each duct is lined with two layers of epithelial cells. The inner layer, facing the ductal lumen, is comprised of cuboidal epithelial cells which can differentiate into lactocytes (milk secreting cells) as needed during the PLC. The basal/outer layer of cells contains myoepithelia which exhibit smooth muscle cell properties and tightly surround the luminal cells.

Recent evidence suggests that the basal layer likely also contains a bi-potent mammary stem cell population (Visvader, 2009). Several studies have suggested this mammary stem cell population can be accurately characterized with a  $CD49f^{high}$  (Integrin  $\alpha 6$ )/ $CD29^{+}$  (Integrin  $\beta 1$ )/ $CD24^{low}$  (Cluster of Differentiation 24) profile and can differentiate into both luminal and myoepithelial cells (Asselin-Labat et al., 2010; Joshi et al., 2010; Visvader, 2009).

Since the formulation of the cancer stem cell theory, such populations have received much attention (Polyak and Weinberg, 2009). Although many lines of evidence suggest the plausibility of such a cell type being the cause of breast cancer progression, the mechanisms involved in regulating mammary stem cell growth and differentiation have yet to be clearly demonstrated (Smalley and Ashworth, 2003).

While the exact origins of breast cancer remain opaque (Erbas et al., 2006; Wren, 2007), the majority of invasive and non-invasive malignant growths arise from epithelial cells that line ducts. These cuboidal epithelia are highly organized with regard to both tissue structure and individual cell polarity (Visvader, 2009). Epithelial polarity, i.e. the asymmetric distribution of proteins within a cell, has long been appreciated as a barrier to growth and de-differentiation (Royer and Lu, 2011). Only more recently have key mediators of polarity been discovered to play essential roles in promoting differentiation and growth (Grunert et al., 2003; Halder and Johnson, 2011; Handler, 1989).

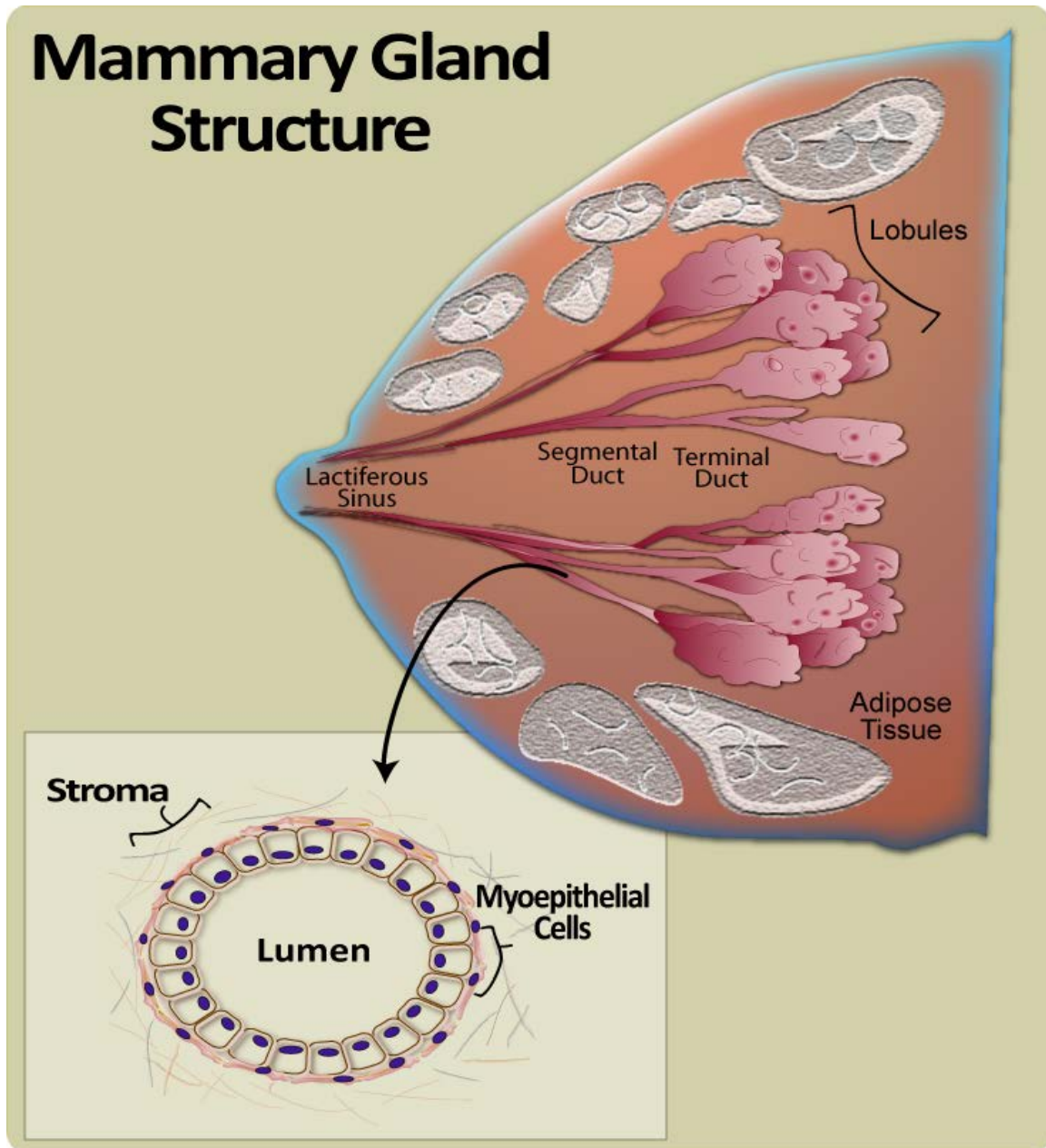
Prevention of breast cancer will be aided by the identification of the earliest initiating events which lead to aberrant growth within the mammary duct. The resting state, i.e. non PLC state, of the mammary gland consists of highly polarized cells which together form a polarized tissue. It is necessary to appreciate the intracellular organization of ductal epithelial cells since changes to intracellular polarity are intimately interrelated to changes in tissue structure. These cells maintain their intrinsic ability to undergo epithelial to mesenchymal transitions (EMTs). EMTs have long been appreciated as a hallmark of cancer (Hanahan and Weinberg, 2000). While the genetic and molecular profiles of epithelial and mesenchymal cells have been elucidated (2012), how growth factor controlled changes in gene expression are coordinated with structural remodeling of the cell remains unclear.

Although measurements of changes in gene expression may indicate whether a cell is epithelial or mesenchymal, they do not explain how those genetic changes were initiated. In order to identify these initiating events, the order of cause and effect must be identified. One example involves the adherens junction associated protein  $\beta$ -catenin.  $\beta$ -catenin is sequestered at adherens junctions following the establishment of cohesion via the transmembrane protein E-cadherin. Although  $\beta$ -catenin forms an integral part of the adherens junction, this protein can also enter the nucleus. Nuclear  $\beta$ -catenin can drive expression of pro-growth genes such as c-



Myc, but this requires both the disruption of  $\beta$ -catenin from adherens junctions and inhibition of Wnt's negative regulation (MacDonald et al., 2009). Wnt signaling must therefore be disrupted prior to release of  $\beta$ -catenin from adherens junctions in order to effect growth. In this way extracellular signals begin the process of transformation from a quiescent epithelial cell to a mesenchymal state. The outer edge of what is currently understood about cancer signaling includes the exact mechanisms required to transmit extracellular signals into changes in gene expression. For example, it remains unclear how epithelial cells convert cell-cell contact into growth inhibitory signaling. Examples such as the  $\beta$ -catenin pathway provide clues as to how extracellular signals may be converted into changes in gene expression. The further question of how these regulatory pathways are hijacked during tumorigenesis remains to be answered. Epithelial organization is a barrier to aberrant growth (Royer and Lu, 2011). Mediators of epithelial polarity are therefore relevant to the discussion of breast cancer progression. This topic will be considered in the next section.

# Mammary Gland Structure



**Figure 1-3 Gross Anatomy of the Breast.**

The breast is comprised of secretory glandular tissue surrounded by adipose tissue which is supported by a network of fibrous connective tissue. Each lobe contains several lobules which in turn are made up of many alveoli. A network of ductal tissues serve to connect the glandular secretory tissues to the nipple, mediating milk transport and storage during lactation. The ductal network is composed of a bi-layer of epithelial cells. Facing the lumen of the duct are cuboidal epithelial cells capable of differentiating into lactogenic cells, and facing the stromal surface are myoepithelial cells that display characteristics of smooth muscle tissue.

#### 1.4 Epithelial Polarity Complexes and their Roles in Growth and Quiescence

Epithelial cell structure and internal organization is disrupted early during mammary tumorigenesis. While the mechanisms involved in initiating these events remain unclear, developmental models of mammary organogenesis have provided clues as to how cells establish and maintain their structure and tissue specific functions (Schmeichel and Bissell, 2003). Glandular epithelia contain a lumen-facing apical domain and basement membrane-facing basolateral domain. This asymmetric organization is termed apical-basal cell polarity and is a characteristic trait of epithelial cells. Cell-cell cohesion is mediated by the junctional complexes termed tight junctions (TJ), adherens junctions (AJ), gap junctions, and desmosomes (Farquhar and Palade, 1963). These junctions are composed of a range of proteins which are organized into three classes. The first class includes transmembrane proteins which mediate interactions between neighboring cells. The second class are comprised of cytoplasmic, peripherally associated, proteins which link the transmembrane proteins to actin filaments or other cytoplasmic proteins. The third class includes a variety of signaling proteins whose functions range from junction assembly to gene transcription (Itoh and Bissell, 2003; Shin et al., 2006). In polarized epithelia these complexes are asymmetrically localized on the cytoplasmic face of the cell-cell contacts.

The TJ complex serves several critical functions, including controlling selective paracellular permeability across epithelial cell sheets, and maintaining a barrier to diffusion of lipids and proteins between the apical and basolateral domains of the plasma membrane. The TJ complex also directly organizes signaling pathways that regulate the cell-cycle (Schneeberger and Lynch, 1992; Shin et al., 2006; Spadaro et al., 2012; Spring, 1998). In mammary epithelia, the composition of the TJ changes dramatically during the PLC. Freeze-fracture electron microscopy images revealed a decrease in tight junction strand number, organization, and branched networks between pregnancy and lactation (Morgan and Wooding, 1982). The structural and functional

integrity of the mammary tight junction is regulated by progesterone, prolactin, and glucocorticoids through unknown mechanisms (Nguyen and Neville, 1998; Stelwagen et al., 1998).

The best characterized TJ associated transmembrane proteins are occludin, claudin, and junctional adhesion molecule (JAM). Occludin was the first TJ protein to be discovered and is thought to incorporate directly into TJ strands (Furuse et al., 1993). The claudins comprise a family of over 20 proteins that localize exclusively to the TJ and serve as the primary component of the stand (Furuse et al., 1998; Tsukita et al., 2001). Five JAM proteins have been identified that all localize to tight junctions in both epithelial and endothelial cells (Martín-Padura et al., 1998). JAM proteins are the first components of the TJ to concentrate at cell contact sites and may therefore function to cue TJ formation (Suzuki et al., 2002).

Many cytoplasmic proteins localize to the tight and adherens junctions. Among these are three main complexes that have emerged as key signaling nodes in the generation and maintenance of apical-basal polarity. These complexes are the Crumb (Crb), Partition defective (Par), and Scribble complexes which are asymmetrically distributed within polarized epithelial cells. These complexes achieve and maintain polarity by integrating signals from transmembrane proteins to intracellular signaling that controls cell shape and gene expression (Assémat et al., 2008).

The Crb and Par complexes dynamically interplay with each other to perform three key functions. First, they maintain the circumferential belt located between TJ complexes, thus defining the apical domain (Schneeberger and Lynch, 1992). Second, they organize the secretory and endocytic systems by regulating vectorial processes of plasma membrane (PM) associated proteins and lipids (Rodriguez-Boulán et al., 2005). Third, these complexes regulate the cytoskeleton by adapting extracellular cues to small GTPases (Lin et al., 2000).

The Crumb complex was first discovered in *Drosophila melanogaster* as the most apically localized of the three complexes (Tepass et al., 1990). The Crumb complex contains, in

addition to Crb, Protein Associated with Lin7 1 (PALS1), and Pals1 Associated Tight Junction protein (PATJ). While Crb is a transmembrane protein, PALS1 and PATJ are cytosolic scaffolding proteins (Assémat et al., 2008). *Drosophila* embryos with mosaic inactivation of Crb expression display apical-basal polarity defects in affected epithelial cells (Bulgakova and Knust, 2009). Overexpression of Crb expands the apical domain of *Drosophila* epithelia at the expense of the basolateral domain (Wodarz et al., 1995). In mammals, three Crb genes regulate apical polarity. Crb1 and Crb2 are expressed in brain and retina tissues, whereas Crb3 is expressed in skeletal and all epithelial tissues (Hollander et al., 2001; Lemmers et al., 2004). Loss of Crb3 expression has been correlated with increased tumorigenic potential in mouse epithelial cells. Re-expression of Crb3 was shown to restore cell-cell junctional integrity, and cell polarization, which limited cell motility and metastasis (Karp et al., 2008). Recently, disruption of the Crumb complex was shown to enhance epithelial to mesenchymal transitions, in human mammary epithelia, via loss of negative regulation of the Hippo pathway (Varelas et al., 2010). This pathway is discussed further in section 1.6

The Par complex lies at the interface between the apical and basolateral domains. The Par complex consists of partition defective 6 (Par6), partition defective 3 (Par3), and atypical Protein Kinase C (aPKC). First identified in the nematode *Caenorhabditis elegans* (Watts et al., 1996), the Par complex was the first polarity complex to be implicated in the polarized migration of epithelial cells. Initially, Par6 and aPKC were identified as downstream targets of the Rho family small G-proteins, Cell Division Control protein 42 (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) (Joberty et al., 2000; Lin et al., 2000). The Par complex was next implicated in controlling directional migration subsequent to wounding (Etienne-Manneville and Hall, 2001). In addition to roles in migration, Par complex components have been shown to play key roles in mediating oncogenic growth signals. For example, the mammary oncogene Human Epidermal Growth Factor Receptor 2 (Her2) (a.k.a. ErbB2) requires an interaction with Par6 to induce loss of polarity and to inhibit apoptosis (Aranda et al., 2006). ErbB2 is a receptor tyrosine

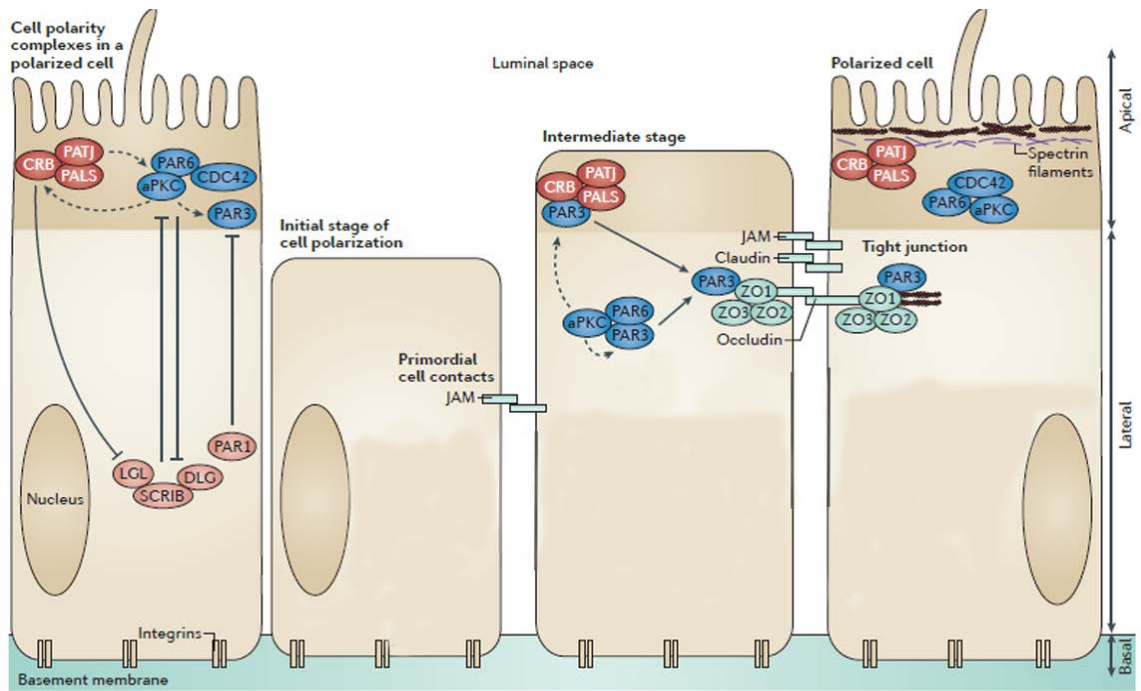
kinase (RTK) which activates canonical mitogen activated protein kinase (MAPK) cascades. MAPK signaling is required for growth of many tumor types, for instance it is required for approximately 30% of mammary tumors (Dhillon et al., 2007; Slamon DJ, 1989). Strikingly, transforming growth factor  $\beta$  (TGF $\beta$ ), a well-established promoter of EMT, also requires an interaction with Par6 to disrupt tight junctions and promote growth (Ozdamar et al., 2005).

As each new layer of regulation is uncovered, the picture of how polarity is maintained gains resolution. Tight junctions provide a physical landmark to establish the identity of the apical and basolateral domains within cells. Tight junctions also allow for selective permeability to tissue structure, and anchor apical polarity complexes. The Crb and Par complexes help maintain the integrity of the apical junctions. Crb and Par complexes also actively inhibit pro-growth pathways by promoting the segregation of agonists and their receptors and by stabilizing cascades mediating cell-cycle arrest (Handler, 1989; Lelièvre, 2010; Tanos and Rodriguez-Boulan, 2008).

Apical polarity regulation also includes the orientation of protein trafficking. Proteins that bind and redirect Crb and Par components away from the plasma membrane provide a mechanism for the disruption of TJ and AJ integrity (Tanos and Rodriguez-Boulan, 2008). This tertiary level of regulation is particularly interesting since the Crb and Par complexes need to be properly localized to mediate junctional stability. By adjusting the trafficking of Crb and Par complexes the integrity of the entire apical domain can be compromised (Tanos and Rodriguez-Boulan, 2008). It remains to be determined whether these traffic-related components are the initiators of polarity disruption during tumor progression.

Polarity complexes have been implicated in both maintaining cellular quiescence and in cancer signaling (Etienne-Manneville, 2008; Shin et al., 2006), thus a significant question is how these complexes can promote or inhibit differentiation. An important aspect in answering this question is understanding how the programing involved in initiating a temporary growth phase, during normal development or wound healing, involves the same modalities that are hijacked

during tumorigenesis. To address these issues, a discussion of two highly relevant signaling pathways (MAPK and Hippo), which mediate the interface between polarity and aberrant growth, are included in the following sections.



**Figure 1-4 Epithelial Polarity**

Epithelial polarity is established by the dynamic interactions of three polarity complexes. The apical-most complex Crumbs (red), is comprised of the transmembrane protein Crumb (Crb), and the cytosolic scaffolding proteins PatJ and Pals1. At the interface between the apical and basolateral domains is the Partition defective (Par) complex (blue) which is comprised of Par6, Par3, and aPKC. Establishment and maintenance of the basolateral domain is mediated by the Scribble complex (pink). The apical-most junctional complexes include the tight and adherens junctions (light blue and purple). These junctions contain transmembrane proteins attached to the cytoskeleton via intermediate cytosolic adaptors. Figure adapted from (Martin-Belmonte and Perez-Moreno, 2012).



### 1.5 MAPK Signaling in Breast Cancer Progression

Approximately 30% of human breast cancers exhibit some deregulation in the MAPK pathway. Aberrant MAPK signaling enables tumors to upregulate proliferation programs while avoiding apoptosis (Cobleigh et al., 1999; Dhillon et al., 2007; Navolanic et al., 2003; Slamon DJ, 1989). The MAPK pathway transmits and amplifies signals involved in cell proliferation through a series of protein kinases. These kinases convert graded inputs into on-or-off outputs to effectively filter out low level signaling noise. The MAPK pathway is activated upon receptor-ligand interactions, such as epidermal growth factor (EGF) binding to epidermal growth factor receptor (EGFR). This signal is either amplified or eliminated by MAPKs through a requirement of the terminal kinase to be dually phosphorylated. Receptor-ligand interactions that are insufficient to mediate full phosphorylation of the terminal protein kinase fail to produce a biological effect (Cobleigh, 1999). The biological function of MAPK signaling is regulated in a spatial-temporal manner. MAPK signaling occurring at the plasma membrane mediates transient, non-genetic signaling, whereas prolonged MAPK signaling occurs in endosomal compartments and results in changes in gene expression (Brown and Sacks, 2009).

Canonical MAPK signaling is initiated upon an agonist binding to a RTK which results in autophosphorylation of the receptors on tyrosine residues. These phosphorylated tyrosines complex with the Src Homology 2 (SH2) domains in adaptors such as Shc and Growth factor receptor bound protein 2 (Grb2). The adaptors then recruit guanine nucleotide exchange factors (GEFs), such as Son of Sevenless (SOS), to the plasma membrane. Recruitment of the GEF brings it in contact with small G proteins that are activated, such as rat sarcoma (Ras). GEFs promote the association of Ras with guanosine triphosphate (GTP) by inducing dissociation of guanosine diphosphate (GDP). Ras, in its GTP-bound form, binds and promotes the activation of the serine/threonine protein kinases A-, B- c-Raf. Rafs phosphorylates/activates MAP/ERK kinases (MEK1/2) which then phosphorylate/activate extracellular signal-regulated kinases

(ERK1/2). If ERK is phosphorylated on both its threonine and tyrosine residues its intrinsic kinase activity is increased several thousand fold. Dually phosphorylated ERK can enter the nucleus, where it forms a ternary transcription complex with serum response factor (SRF) and ETS-like gene 1 (Elk1), which is then competent to drive expression of target genes involved in growth and de-differentiation (Ahn, 1993; Cobb and Goldsmith, 1995; Cobb, 1991; Crews and Erikson, 1992; Kyriakis et al., 1992) (Figure 1-5).

Breast cancers, as well as models of these diseases, can be divided into two groups. These two groups are those that require steroid hormones, such as estrogen for growth and those that do not. The tumor types which do not require steroid hormones for growth are alternatively driven to grow by peptide hormones or growth factors, such as EGF, TGF $\alpha$  and TGF $\beta$ . These factors directly activate the MAPK pathway by the mechanism described above (Das and Vonderhaar, 1996). In addition to canonical agonist/receptor MAPK activation, Ras activation may be achieved without agonists where receptors are constitutively active as in the case of ErbB2/Her2 tumors (Janes et al., 1994).

The subset of tumor types which require estrogen for growth can often be effectively treated with selective estrogen receptor modulators (i.e. Tamoxifen) which inhibit estrogen action and inhibitors of estrogen synthesis (i.e. aromatase inhibitors) (Johnston, 2010). Although estrogen dependent mammary cancer cells such as MCF-7 can grow independently of MAPK activation (Addeo et al., 1996; Cicatiello et al., 2000), there are several ways in which estrogen dependent tumor cells interface with the MAPK pathway. These modes of crosstalk become especially relevant when treatment of estrogen receptor positive (ER+) tumor types results in recurrence of tumors resistant to estrogen-targeted therapy (Arpino et al., 2005; Dent et al., 2007). Estradiol (E2), the dominant member of the estrogen family of sex hormones, has been shown to stimulate the MAPK cascade in breast cancer cells via both the classic genomic and non-genomic ER pathways. Within the classic genomic pathway of ER activation, E2 promotes increased levels of several growth factors and their receptors which signal through the MAPK pathway.

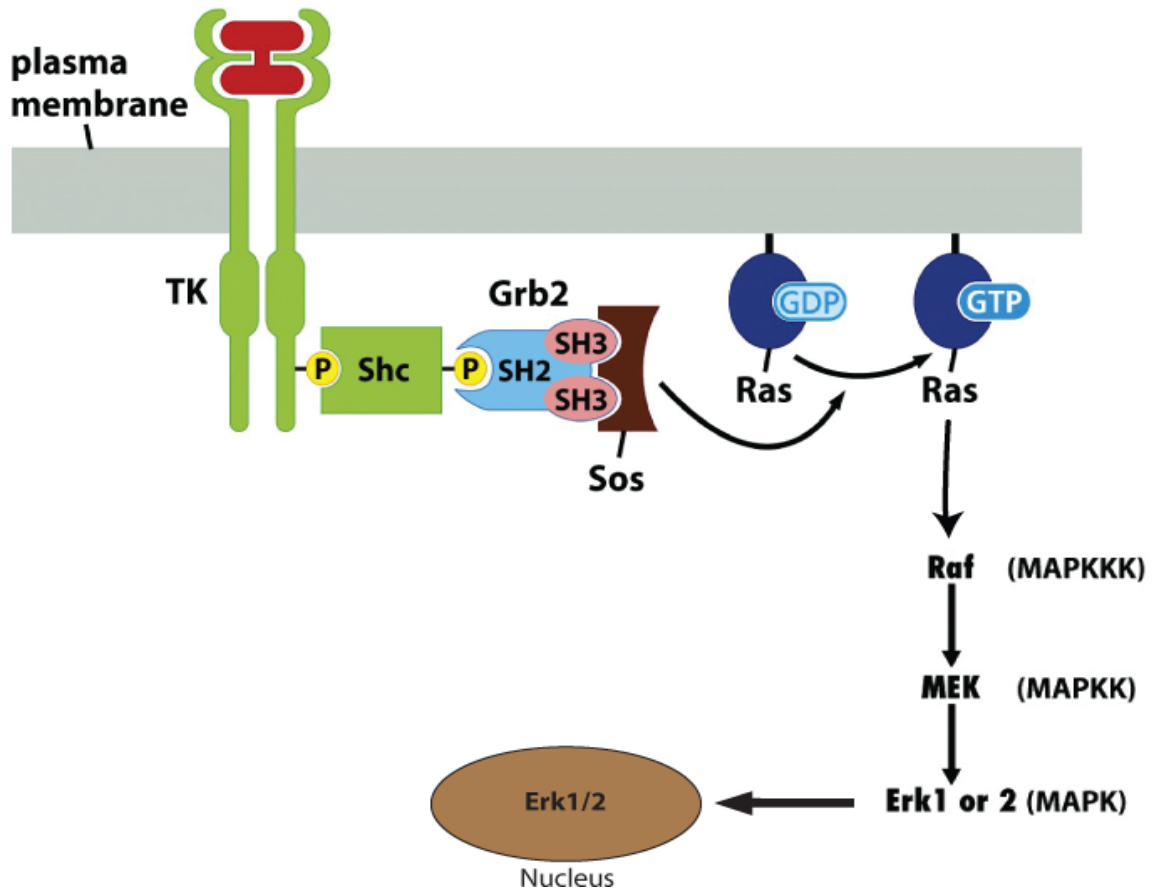
Therefore disruption of the MAPK pathway inhibits the ability of E2 to drive cancer cell proliferation (Clark et al., 1996; Lobenhofer et al., 2000). Estradiol has been observed to affect many MAPK-related changes within cancer cells which are considered non-genomic given that the effects are observed within hours or even minutes of stimulation. These effects include prolactin secretion (Norfleet et al., 2000), inositol phosphate production (Razandi et al., 1999), activation of G $\alpha$  proteins (Razandi et al., 2000), and Shc phosphorylation (Migliaccio et al., 1998). Thus in both hormone dependent and independent breast cancers, MAPK signaling plays a key role in promoting proliferation.

Among the hormone non-responsive tumors, “triple negative” breast cancers are particularly lethal. Triple negative breast cancers present with no estrogen/progesterone receptor (PR) and do not upregulate Her2 expression (ER-/PR-/Her2-). Triple negative tumors are found within 12-17% of women with breast cancer. This tumor type cannot be treated with standard endocrine therapies or with anti-Her2 receptor targeting therapies, and thus have very poor outcomes (Dent et al., 2007; Foulkes et al., 2010; Hudis and Gianni, 2011; Shastry and Yardley, 2013). Much effort is in progress to develop new therapies that focus on the MAPK pathway in triple negative breast cancers (Bayraktar and Glück, 2013; Shastry and Yardley, 2013).

In each of the above mentioned cancer subtypes, there is disruption of normal epithelial polarity. Several reports implicate polarity proteins in both the initiation and progression of tumorigenesis (Aranda et al., 2006; Feigin and Muthuswamy, 2009; Karp et al., 2008; Nolan et al., 2008; Royer and Lu, 2011; Tanos and Rodriguez-Boulan, 2008; Xue et al., 2013). It remains unclear, however, whether loss of polarity and MAPK activation is causal, occurs concomitantly with, or subsequent to, transformation. The order of events which occur as epithelial cells undergo transformation can only be determined once the role of epithelial polarity in promoting or inhibiting MAPK signaling is clarified. While components of epithelial polarity are clearly involved in MAPK signaling (Royer and Lu, 2011), little is known about how polarity proteins switch between promoting polarity and promoting MAPK signaling. Further, mounting evidence

indicates that polarity proteins are not limited to regulation of growth through MAPK signaling (Etienne-Manneville, 2008).

Recently, a universal organ size control mechanism termed the Hippo pathway was discovered (Dong et al., 2007). The Hippo pathway links cell-cell contact to inhibition of growth. This finding not only sheds light on how cell-cell contact effects gene expression but also gives insight into the priority of events which must take place for an EMT to occur. Importantly, several studies indicate that Hippo controlled gene expression patterns change subsequent to loss of apical polarity, thus placing regulators of polarity at the top of the EMT cascade (Bulgakova and Knust, 2009; Feigin and Muthuswamy, 2009; Varelas et al., 2010).



**Figure 1-5 Canonical MAPK signaling pathway**

Receptor tyrosine kinases bind ligands, resulting in autophosphorylation of the receptors on tyrosine residues. Activated receptors complex adaptors such as Shc and Grb2. Shc and Grb2 recruit GEFs, such as SOS, to the plasma membrane, bringing them in contact with the GTPases they activate, for example, Ras. GEFs promote the association of Ras with GTP. Once Ras is activated it binds and promotes the activation of kinases, such as Raf-1. Raf phosphorylates MEK which then phosphorylates ERK. ERK translocates to the nucleus where it drives expression of target genes involved in cell proliferation. Figure adapted from (Hanahan and Weinberg, 2000).

## 1.6 Organ Size Control and Yap/Taz Activity in Breast Cancer Progression

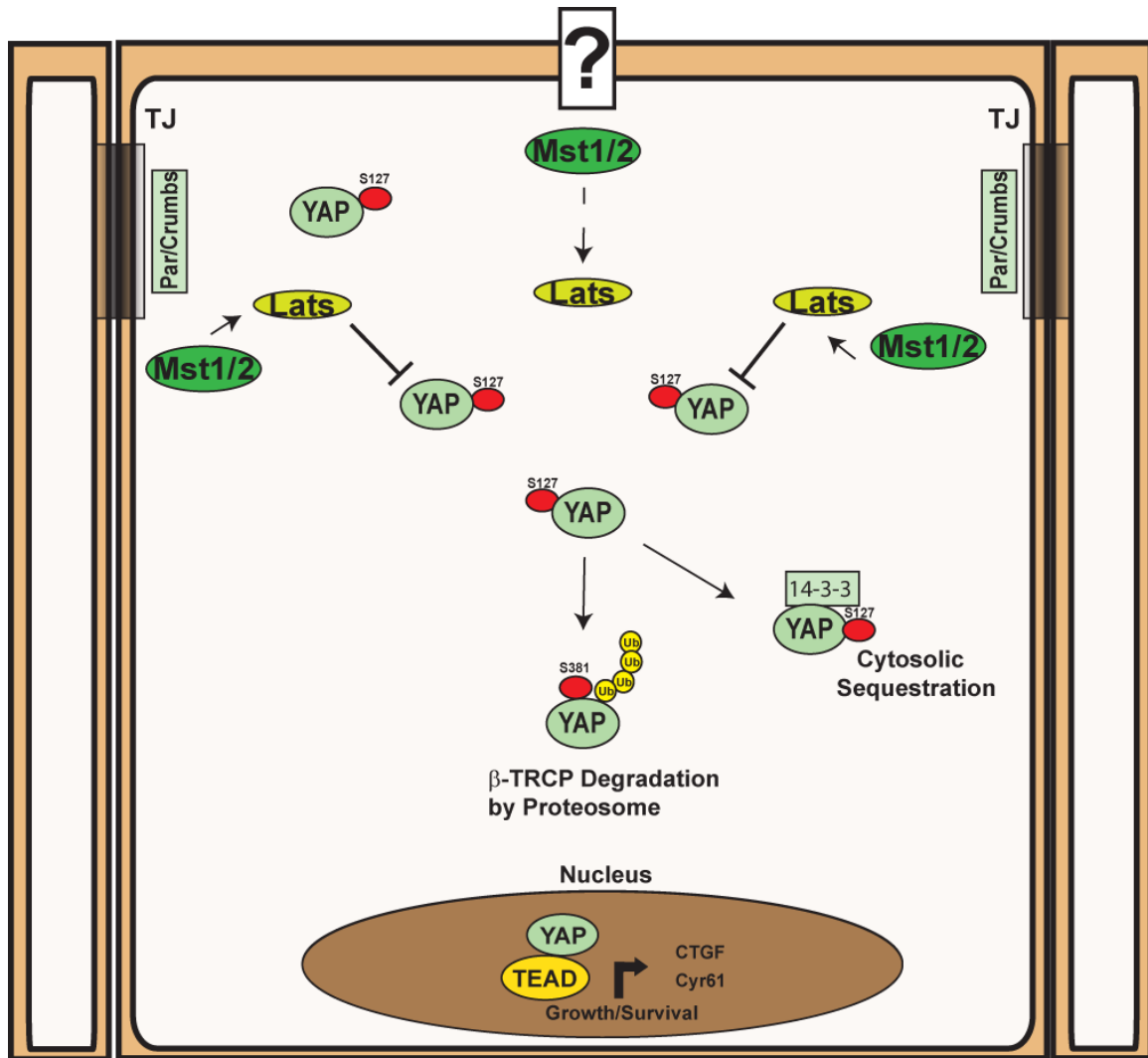
The transcriptional co-activator Yes-associated protein (Yap) and its functional homologue, Transcriptional co-activator with PDZ-binding motif (Taz) have recently become the focus of a great deal of interest (Kango-Singh and Singh, 2009; Zhao et al., 2008a). In the past decade a new signaling pathway, termed Hippo, has been described in which Yap regulates organ size through control of both cell proliferation and apoptosis. Hippo pathway components were originally discovered in *Drosophila* (Harvey et al., 2003; Jia et al., 2003; Justice et al., 1995; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003; Xu et al., 1995). Strikingly, the mammalian homologues of the Hippo pathway are able to rescue the phenotypes of their *Drosophila* mutants *in vivo* (Huang et al., 2005; Lai et al., 2005; Wu et al., 2003) reinforcing the notion of a universal organ size control mechanism conserved throughout metazoans (Dong et al., 2007).

The best understood components of the Hippo pathway include two protein kinases and two transcriptional co-activators. Mammalian Ste20-like protein kinase (Mst1/2) (homologues of Hippo) which are activated following apical junctional formation (Zhao et al., 2007), phosphorylate and activate large tumor suppressor (Lats1/2) protein kinases (Chan et al., 2005). Lats1/2 phosphorylates Yap and Taz, inhibiting their ability to enter the nucleus where they bind TEA-domain containing transcription factors (TEADs). Thus, Hippo signaling couples cell-cell contact to disruption of Yap/Taz transcriptional activity (Dong et al., 2007; Hao et al., 2008; Lei et al., 2008; Oka et al., 2008; Zhao et al., 2007) (Figure 1.6). Yap/Taz transcriptional activity in turn induces migration, invasion, and tumorigenesis in breast cancer cells (Chan et al., 2008; Overholtzer et al., 2006; Zhao et al., 2009; Zhi et al., 2012). Yap/Taz expression has also been shown to induce EMTs (Lei et al., 2008; Overholtzer et al., 2006; Zhao et al., 2008b). Recently Taz was also proposed to confer cancer stem cell related traits on breast cancer cells (Cordenonsi

et al., 2011). Since its genetic amplification has been demonstrated to promote tumor development, Yap is now considered an oncogene (Overholtzer et al., 2006; Zender et al., 2006).

The effectors that function upstream of Mst1/2 are still being elucidated (Zhao et al., 2011b). Recent evidence suggests that Hippo protein kinase activity is regulated on several levels. Cell polarity components including intercellular junctions and polarity complexes, as well as the cytoskeleton, have been demonstrated to regulate Yap/Taz activity (Chan et al., 2011; Schlegelmilch et al., 2011; Silvis et al., 2011; Wada et al., 2011; Wang et al., 2011; Yi et al., 2011; Zhao et al., 2011a; Zhao et al., 2012a). Several lines of evidence suggest that regulators of polarity are inactivated during tumorigenesis (Huang and Muthuswamy, 2010; Javier, 2008; Lee and Vasioukhin, 2008). The Hippo pathway appears to translate the cell-cell contact/establishment of polarity signal into inhibition of growth via negative regulation of Yap/Taz (Genevet and Tapon, 2011; Ota and Sasaki, 2008; Zhao et al., 2007). It is therefore highly plausible that negative regulators of polarity are responsible for inactivation of the Hippo pathway.

With the discovery of the Hippo pathway new insights have emerged linking the disruption of epithelial polarity with changes in gene expression that result in transformation (Boggiano and Fehon, 2012; Genevet and Tapon, 2011; Spadaro et al., 2012). Epithelial cells maintain polarity by the combined functions of junctional complexes (i.e. tight junctions), polarity complexes (i.e. Par and Crb), and polarity adaptor proteins. The correct distribution of apical polarity complexes and their cross-talk with other pathways including Hippo are maintained by apical polarity adaptors (Assémat et al., 2008; Tanos and Rodriguez-Boulán, 2008). The final section of this introduction will therefore focus on the polarity adaptor protein Amot. This includes the role of Amot in regulating apical polarity complexes, and its role in apical trafficking. How polarity adaptor proteins such as Amot are positioned, via disruption of apical polarity, to play a central role during EMTs will also be discussed.



**Figure 1-6 Hippo Signaling in Mammals**

MST1/2 kinases are activated by unknown factors, likely receptors sensing cell-cell contacts and/or apical junctions. Activated MST1/2 phosphorylates Lats1/2 kinases which phosphorylate Yap/Taz. These phosphorylations result in Yap/Taz cytoplasmic sequestration and may also mediate degradation. Yap/Taz proteins upon entering the nucleus bind TEAD transcription factors that in turn drive expression of genes involved in cell proliferation.



### 1.7 The Role of the Polarity Adaptor Protein Amot in Epithelial Polarity and Apical Trafficking

The polarity protein angiomin (Amot) has only recently gained appreciation for its role as a regulator of growth and de-differentiation. Since the historical and currently known functions of Amot are both relatively limited, this section includes a brief discussion of some particularly relevant studies. The 80kDa isoform of Amot (Amot80) was first discovered in 2001. Yeast two-hybrid screen analysis revealed Amot as a binding partner of angiostatin. Amot was initially described as a stimulator of endothelial cell motility (Trojanovsky et al., 2001). Consistent with this report a second group showed that Amot expression was upregulated during chemotaxis-mediated cell motility in neutrophils (Benelli et al., 2001). Interestingly, Amot expression was also shown to be significantly upregulated during the acute inflammatory phase of murine excisional wounds (Roy et al., 2008). These initial reports clearly suggested a role for Amot in inflammation and migration.

A report in 2003 detailed how genetic inactivation of Amot in mice resulted in embryonic lethality due to migration and proliferation defects in the anterior visceral endoderm (Shimono and Behringer, 2003). That same year the Post synaptic density protein/Drosophila disc large/Zonula occludens-1 protein (PDZ) binding domain on the extreme C-termini of Amot was found to be required to facilitate chemotactic migration. Genetic expression of the PDZ mutant of Amot80, but not WT Amot80, resulted in embryonic death of mice due to inhibition of endothelial cell migration into the neuroectoderm and intersomitic regions (Levchenko et al., 2003).

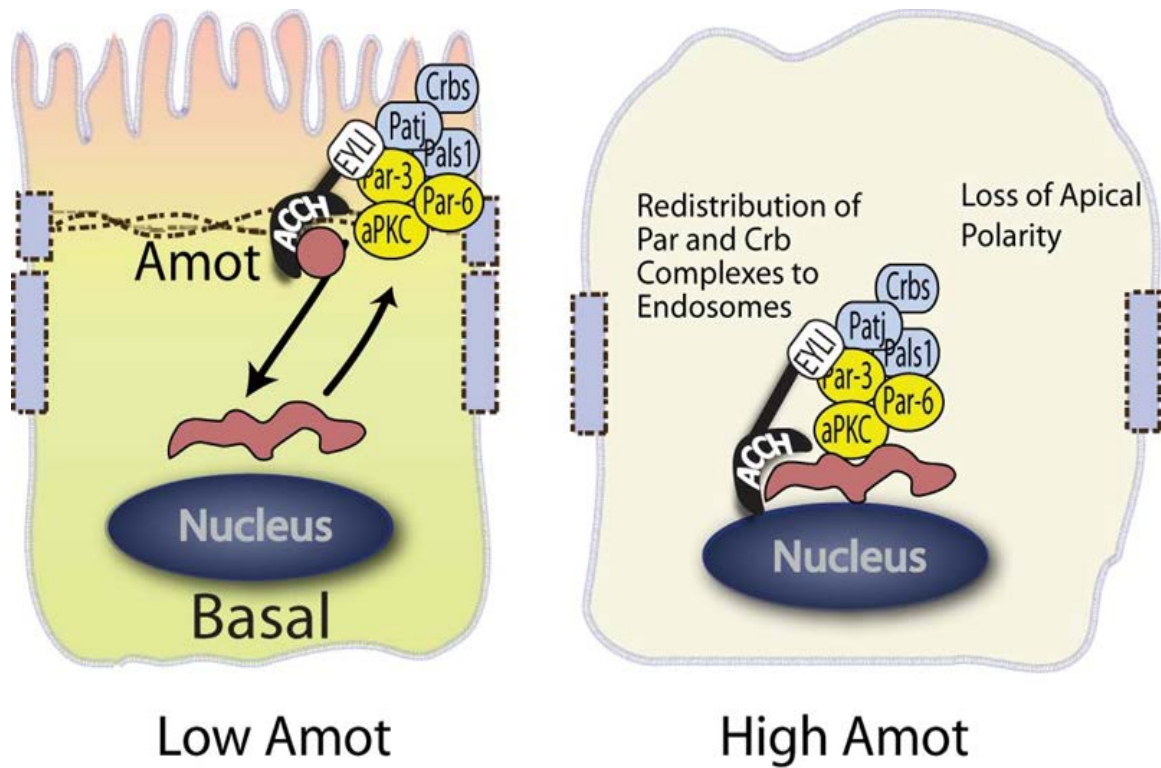
The first report describing a tumorigenic role for Amot came in 2004. Amot expression in mouse aortic endothelial cells was shown to result in tumor growth with significant invasion of cells into muscle tissue in mice (Levchenko et al., 2004). In 2005 a splice isoform of Amot was described which produced a 130kDa protein product (Amot130) (Bratt et al., 2005). Until that point only the 80kDa (Amot80) gene product was known to exist. Previous work had expanded

the family of Amot proteins to include AmotL1 and AmotL2 (Bratt et al., 2002) which were also shown to produce alternatively spliced isoforms (Moreau et al., 2005). It was not until several years later that differential roles for Amot130 and Amot80 began to surface (Roudier et al., 2009). The ratio of Amot80 to Amot130 was shown to regulate the migration or stabilization of endothelial cells. Amot80 expression was demonstrated to promote migration while Amot130 expression promoted stabilization (Ernkvist et al., 2008). Further studies of Amot130 suggested it also had anti-proliferative functions (Bratt et al., 2005; Ernkvist et al., 2006).

Amot was first proposed to be a potential therapeutic target for breast cancer in 2006. The Mansel group demonstrated that breast cancer tissues express significantly higher levels of Amot mRNA than normal mammary tissues. They also showed that Amot mRNA transcript expression increased with tumor grade and correlated its expression with shorter overall patient survival (Jiang et al., 2006). That same year Amot80 expression was shown to regulate epithelial polarity (Wells et al., 2006). This work focused on establishing a network of Amot80 interactions with apical polarity regulators and demonstrated that Amot80 expression results in redistribution of members of both the Par and Crb complexes away from apical junctions. Amot80 expression was thus shown to disrupt apical polarity. This work also established a role for apical polarity adaptors in regulation of small G proteins (Wells et al., 2006). The ability of Amot80 to regulate GTPases has since been well established (Aase et al., 2007; Ernkvist et al., 2009; Garnaas et al., 2008; Yi et al., 2011).

Amot80 has also been shown to induce the internalization of polarity complexes. Amot80 contains a membrane binding domain that selectively recognizes membranes containing monophosphorylated phosphatidylinositols and cholesterol. Membranes to which this domain binds in cells also contain Rab11 and Arf6, markers of juxtanuclear recycling endosomal compartments (Heller et al., 2010). The functional consequence of Amot80 mediated internalization of polarity complexes on cell growth has yet to be demonstrated.

Recently, several studies have established an interaction between Amot family members and Yap/Taz regulation (Chan et al., 2011; Oka et al., 2012; Paramasivam et al., 2011; Varelas et al., 2010; Wang et al., 2011; Zhao et al., 2011a). This is consistent with Amot playing a dominant role in maintaining apical polarity in epithelial cells. These studies, however, focused on Amot130, AmotL1, and AmotL2 and described them collectively as negative regulators of Yap/Taz. To date, no studies have investigated how the ability of Amot80 to regulate polarity may underlie Hippo signaling.



**Figure 1-7 Amot80 Negative Regulation of Apical Polarity in Epithelial Cells**

Amot80 interacts with members of the Crb and Par complexes via its extreme C-terminal PDZ-binding domain. Amot80 also contains an N-terminal ACCH domain which selectively recognizes endosomal compartments. Amot80 expression dominantly redirects apical polarity complexes away from apical junctions, thus disrupting apical polarity.

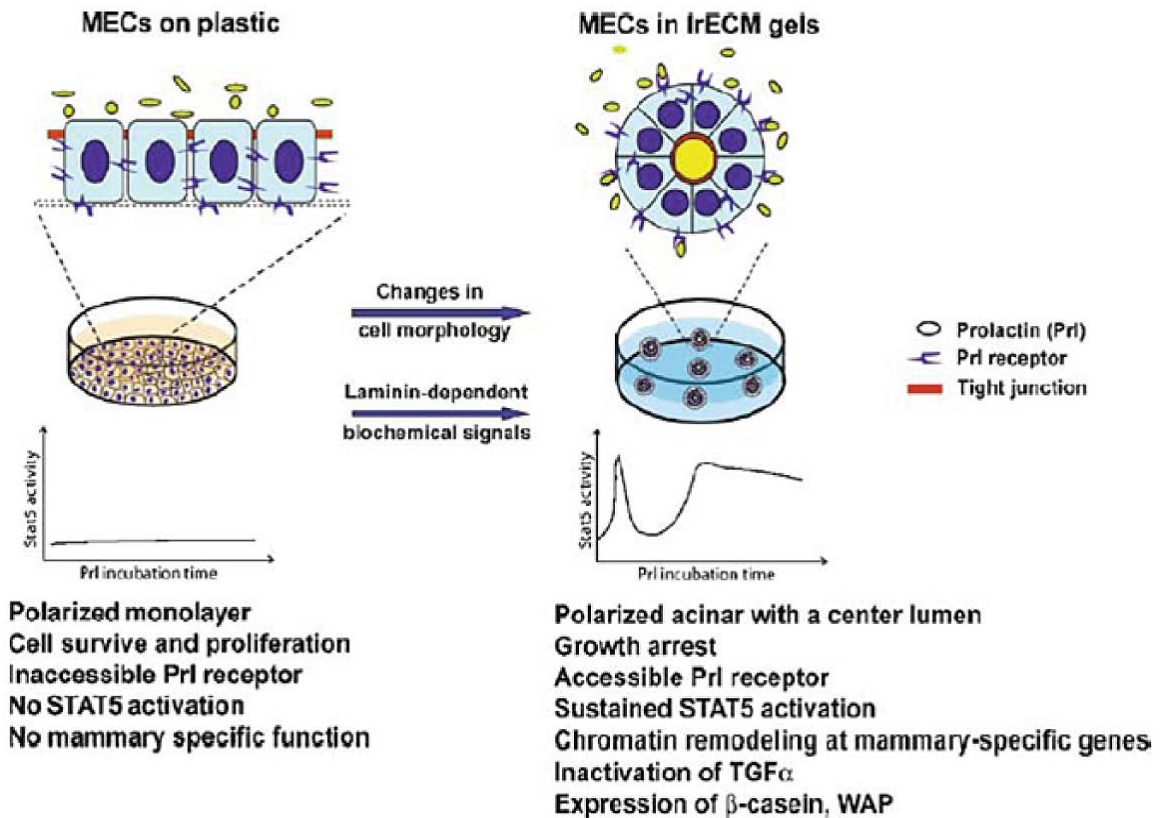
## 1.8 The Use of Mammary Cell Lines and 3D Models in this Study

Mammary tumorigenesis is triggered by a wide range of hormonal and growth factor mediated pathways (de Waard, 1969). There are also a wide range of mammary tumor types, each with differing metastatic potentials and sensitivity to therapeutic treatments (Russo J, 1992). It is therefore necessary to exploit specific models of breast cancer that accurately reflect the tumor types under investigation.

Fortunately much work has been done to establish relevant mammary cancer cell lines which mainly reflect the tumor types from which they were derived (Kao et al., 2009; Neve et al., 2006). This study used several different mammary cell types, both tumorigenic and non-tumorigenic. Tumorigenic cell lines were chosen to represent a range of tumor types, as well as diagnostic markers for treatment e.g. ER-/PR-/Her2-. The luminal, ER+, MCF7 cell line was chosen as a representative of the hormone receptor positive, IDC tumor type. The MCF7 cell line is used widely as a model to study ER+ hormone therapy treatment and acquisition of estrogen resistance. MCF7 cells also represent a common breast cancer type with readily available hormonal treatments. Finally, this cell type also is a model for investigation into the mechanisms involved in hormone independent tumor recurrence. The ER-/PR-/Her2+ luminal cell line SKBR3 was also derived from an adenocarcinoma but given its Her2 expression status this cell line represents a tumor type for which anti-Her2 treatments are available. SKBR3 cells represent a non-hormonal tumor type which has upregulated Her2 expression and therefore specific treatments are available. MDA-MB-468 cells represent the triple negative adenocarcinoma tumor type which lack hormone dependence for growth and do not have upregulated Her2 expression and therefore present a significant challenge for treatment (Hudis and Gianni, 2011). Finally, a non-tumorigenic mammary epithelial cell line was used as a model for normal breast tissue. The non-tumorigenic MCF10A cell line was generated from a reduction mammoplasty, spontaneously

immortalized, but is non-transformed, and does not form tumors in mice. MCF10A cells provide a unique tool to study normal mammary ductal function (Debnath and Brugge, 2005).

Micro-environmental cues, vital to mammary organogenesis, are lost under standard two-dimensional plastic substrata culturing conditions (Barcellos-Hoff et al., 1989; Bissell et al., 1982; Xu et al., 2009). Non-malignant human mammary epithelial cells can be quickly distinguished from their malignant counterparts when grown in a laminin-rich extracellular matrix (lrECM) commercially available as Matrigel<sup>TM</sup> (Petersen et al., 1992). Non-malignant cells, such as MCF10As, when grown in a lrECM, display a temporary growth phase followed by formation of a polarized, growth arrested structure designated acini (Muthuswamy et al., 2001). Acini accurately model the genetic expression profiles of normal ductal tissues *in vivo*, and remarkably, are able to secrete milk proteins when stimulated (Figure 1-8) (Debnath and Brugge, 2005; Debnath et al., 2003; Kenny et al., 2007). Polarized, functional acini provide a powerful model to study the role of polarity adaptors in a physiologically relevant system.



**Figure 1-8 3D Growth of Non-Malignant Mammary Epithelial Cells**

Non-malignant mammary epithelial cells grown on traditional 2D plastic substrata organize into a polarized monolayer with no mammary specific functions. When grown on a laminin rich extracellular matrix, cells display a brief proliferation phase where they form a solid sphere of cells. This is followed by a period of apoptosis of luminal cells resulting in luminal clearing. Structures, at this point termed 'acini,' display polarized, growth arrested cells, which are functionally and genetically similar to mammary ductal epithelia *in vivo*. Figure adapted from (Xu et al., 2009).

## 1.9 Rationale and Central Hypothesis

The overarching goal of this dissertation is to understand the function of Amot family polarity adapters in general, and Amot80 in particular, in the sequence of tumorigenic events of mammary tumorigenesis. The majority of tumors originate from epithelial cells. Epithelial to mesenchymal transition is a hallmark of cancer and is required for tumor metastasis (May et al., 2011). Epithelial cells contain the innate ability to undergo EMT during both organogenesis and wound healing. These pathways, which coordinate loss of polarity with growth and migration are poorly understood, and are prime pathways for hijacking during cancer progression (Tanos and Rodriguez-Boulan, 2008).

This dissertation aims at furthering our understanding of how epithelial cells are able to repurpose apical polarity complexes, normally involved in maintaining quiescence, to promote growth. Expression of Amot is significantly upregulated in response to inflammatory signaling subsequent to cutaneous wounding (Roy et al., 2008), and is required for cell migration during embryogenesis (Aase et al., 2007). These data suggest roles in both organogenesis and wound healing. Amot has been shown to dominantly bind to and redistribute members of both the Crb and Par complexes (Wells et al., 2006). The implications of this redistribution in mammary epithelia is a focus of this dissertation given that Amot expression has been shown to correlate with tumor grade, metastatic potential, and poor patient survival in breast cancer patients (Jiang et al., 2006).

The effect of Amot80 induced redistribution of polarity components is specifically investigated within the context of two cancer-related signaling pathways. The first line of investigation is focused on the MAPK pathway. This pathway is aberrantly activated in 30% of mammary tumors (Jiang et al., 2006) and therefore represents a distinct challenge to treatment. The second line of investigation focuses on the newly discovered mediators of organ size control, the transcriptional co-activators Yap/Taz. Yap is an oncogene overexpressed in breast cancers



and its regulation in mammalian cells remains unclear (Overholtzer et al., 2006; Zender et al., 2006).

The central hypothesis of this dissertation is that Amot80 coordinates the loss of apical polarity with activation of MAPK pathways and disruption of Hippo signaling. This hypothesis was tested by studying three aims: 1) Determine if Amot80 expression was sufficient to enhance proliferation of mammary cells via MAPK and/or Yap/Taz activation. 2) Examine the requirements of Amot expression for mammary cancer cell survival. 3) Investigate the relative contributions of Amot family members to growth and differentiation alone and in combination with Amot80.

## **CHAPTER 2. MATERIALS AND METHODS**

## 2.1 GENERAL CELL CULTURE METHODS

All cell lines were cultured at 37°C with 5% CO<sub>2</sub>. Cell lines were passaged as follows: Media was aspirated from cells. Cells were washed with pre-warmed phosphate buffered saline (PBS). Cells were covered with 2.5g/L trypsin (Sigma) combined with EDTA (Sigma) (5mM final) until dissociated from plastic. An equal volume of media, as that used to trypsinize cells, was used to collect cells. Cells were transferred to a 15mL conical vial and centrifuged at 0.4Xg for 1.5 minutes. Cell pellets were resuspended in 1ml media with a p1000 before further dilutions or cell counting.

MDA-MB-436 and MDA-MB-231 cells were cultured in L15 with 10% FBS. HCC1937, MCF7, SKBR3, and 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). BT474 and ZR75 cells were cultured in PRMI with 10% FBS. MCF10A cells were cultured in equal parts Hams-F12/DMEM supplemented with 5% horse serum, 0.5µg/mL hydrocortisone, 10µg/mL human insulin, 20ng/mL epidermal growth factor (EGF), and 100ng/mL cholera toxin. MCF10A cells grown on IrECM (Matrigel™) were incubated with "Assay" media containing: per 500mL, 50:50 DMEM:HAMS F12, 10mL Horse serum, 250µl Hydrocortisone (1mg/mL), 50µl Cholera Toxin (1mg/mL), 500µl Insulin (10mg/mL). All cell lines were cultured with 1% penicillin/streptomycin. MDA-MB-436, 231, and 468 as well as HS578T, T47D, and SKBR3 cells were gifts from H. Nakshatri. B. Herbert provided HCC1937, MCF10A, BT474, and ZR75 cells. L. Quilliam supplied MCF7 cells and 293T cells were purchased from American Type Culture Collection (ATCC).

## 2.2 GENERATION OF STABLE CELL LINES USING LENTI-VIRUS

Cells were generated which stably expressed indicated proteins or shRNAs by the following method: Day 1) 3-4 million 293T cells were seeded into a 10cm<sup>2</sup> dish, before noon. This number was empirically determined based on health of the 293T cells. One 10cm dish was required per target transfected DNA. Day 2) Transient transfection of packaging 293T cells was carried out ~12 hours later. Each target DNA construct required the following; 1ml Optimem, 6µg VSVG, 5µg pRSV-Rev, 10µg pRRE, 20µg Target DNA construct, and 25µl PEI (2µg/mL). Transient Polyethylenimine (PEI) transfections were carried out as described below (section 2.5). Day 3) Transfection media was removed, in the morning, and replaced with 7mL of 293T media. Target cell lines were plated for infection. 3X10<sup>5</sup> cells were split into a 6cm<sup>2</sup> dish per condition. If infection of more than one construct was desired, 3X10<sup>5</sup> cells were split into a 10cm dish. If Amot silencing was desired 6X10<sup>5</sup> cells were split into a 6cm dish. Day 4) In the morning, viral media was removed from 293T cells, filtered through a 0.45 µm filter before 2µl polybrene (5mg/mL) was added per mL of viral media. 7mL of 293T media was added to viral producing cells if second round of viral harvesting was desired. Viral media was then added to target cell lines and allowed to infect for at least 6 hours prior to being replaced with appropriate cell line specific media.

## 2.3 GENERATION OF STABLE CELL LINES USING RETRO-VIRUS

Stable expression of the indicated genes or shRNAs was achieved with retro-virus as follows: Day 1) 4 million 293 cells stably expressing Moloney murine leukemia virus gag/pol (293GP) cells were seeded into a 10cm dish, before noon. This number was empirically determined based on health of the 293GP cells. One 10cm dish was required per target

transfected DNA. Day 2) Transient transfection of 293GP cells was carried out ~12 hours later. Each target DNA construct required the following; 1ml Optimem, 20µg VSVG, 20µg Target DNA construct, and 25µl PEI (2µg/mL). Day 3) Transfection media was removed, in the morning, and replaced with 7mL of 293GP media. Target cell lines were split out.  $3 \times 10^5$  cells were split into a 6cm dish per condition. If infection of more than one construct was desired,  $3 \times 10^5$  cells were split into a 10cm dish. If Amot silencing was desired  $6 \times 10^5$  cells were split into a 6cm dish. Day 4) In the morning, viral media was removed from 293GP cells, filtered through a 0.45 µm filter before 2µl polybrene (5mg/mL) was added per mL of viral media. 7mL of 293GP media was added to viral producing cells if second round of viral harvesting was desired. Viral media was then added to target cell lines and allowed to infect for at least 6 hours before being replaced with appropriate cell line specific media.

## 2.4 PLASMIDS, ANTIBODIES, AND CELL LYSIS BUFFERS

Plasmids: Generation of DsRed-Amot80, CFP-Amot80, YFP-Amot 80, 3XFlag-Amot80, CFP-Amot80ΔC, and YFP-Amot80ΔC, 3XFlag-Yap, 2XMyc-Amot80, 2XMyc -Amot130, 2XMyc -AmotL1, and 2XMyc -AmotL2 YFP AmotL1, and YFP-Amot130 were described previously (Colwill et al., 2006; Heller et al., 2010; Wells et al., 2006). 5XGal4-luc, Gal4-TEAD4, CA-Ras (G12V) mutants and ELK-1 reporter (gifts from L. Quilliam), SRE-luciferase and TK-Renilla (Clontech), and psRSVRev, pMDLg-RRE, and pCMV-VSVG (Addgene) were also used.

Antibodies: MEK1/2, MEK1/2 (S217/S221), ERK1/2, ERK1/2 (Y202/Y204; Cell Signaling), Raf1, Raf1 (Y340/Y341; Santa Cruz), GFP (Invitrogen), and HA (12CA5) at 1:1,000, Amot 1:800 (in house) AmotL1 1:100 (in house), Myc (9E10) 1:1000 Flag (Sigma, F3165) 1:10000, Flag (Sigma, F7425) 1:1000, Myc (Cell Signaling, 2272S) 1:1000, YFP/GFP

(Invitrogen, A6455) 1:1000, GAPDH (Millipore, MAB374) 1:5000, YAP1 (Abnova, H00010413-MO1) 1:1000, p-YAP-S127 (Cell Signaling, 4911S) 1:1000. PCNA (Santa Cruz) 1:1000. TAZ (Santa Cruz) 1:1000. SNRNP70 (Santa Cruz) 1:1000.

Cell lysis buffers: Two distinct lysis buffers were used in this study. PLC lysis buffer was used for immunoprecipitations and extraction of proteins from cells grown in Matrigel™. Radioimmunoprecipitation assay buffer (RIPA) buffer was used for extraction of proteins from cells grown on plastic and in Matrigel™. PLC lysis buffer is a solution of: 50mM HEPES (pH 7.5), 150mM NaCl, 10% Glycerol, 1% Triton X-100, 1.5mM MgCl<sub>2</sub>, and 1mM EGTA. RIPA lysis buffer solution contains: 25mL of 1M Tris pH 8.0, 2mL of 0.5M EDTA, 5mL of Triton X-100 (1% final), 5mL 10% SDS (0.1% final), 15mL 5M NaCl, total volume was brought up to 500mL with ddH<sub>2</sub>O. Cell lysis buffers were supplemented with protease inhibitor cocktail (Sigma), 1mM/L NaVO<sub>4</sub>, and 10mM/L NaF.

## 2.5 TRANSIENT TRANSFECTION METHODS

Polyethylenimine (PEI) was used to transiently transfect cells as follows: Target cells were plated based on assay requirements (i.e.  $4 \times 10^6$  293T cells for lentiviral packaging). Cells were allowed at least 18 hours to adhere before transfection. Total transfection volumes were scaled according to plate size (i.e. 10cm plate total transfection volume approximately 1mL). Into an appropriate volume of Optimem (Invitrogen) was added desired construct(s). While vortexing, an equal volume of 2μg/mL PEI was added to optimum/DNA solution (i.e. 5μl PEI for 5ug DNA). No more than 25μl of 2μg/mL PEI was added. Transfection solutions were allowed to incubate for 5 minutes at room temperature before adding to target cells. Transfection solutions were added to cells covered in the appropriate media.

## 2.6 IMMUNOBLOT PROCEDURE AND ANALYSIS

Resolution of proteins was achieved via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as follows. Cells were removed from incubation and placed on ice. Media on cells was removed by aspiration. Cells were washed in cold PBS. RIPA or PLC lysis buffer (described above) was added directly to cells on plates. Cells were scraped and transferred to 1.5mL tubes, pipetted several times to ensure disruption of aggregates then transferred to dry ice for 5 minutes. Cells were thawed on wet ice before a 10 minute centrifugation at 20,817Xg at 4°C. Protein concentrations of clarified lysates were determined by bicinchoninic acid (BCA) (Bio-Rad) assay. Normalized protein concentrations from lysates were boiled in sample buffer and loaded onto 8% polyacrylamide gels. Gels were run at 150V for 1 hour. Proteins in gels were transferred to Protran BA85 nitrocellulose membranes (GE Healthcare Life Sciences) via Genie-Blotter (Idea-Scientific) for 1.5 hours at 12V. Membranes were blocked in a 5% low-fat milk solution for 20 minutes on a rocking platform at room temperature. Membranes were washed 3X in TBST for 5 minutes using an orbital shaker at room temperature. Membranes were transferred to hybridization bags and incubated with 5mls TBST and primary antibody on a nutator for 1 hour at 4°C. Membranes were washed 3X in TBST for 5 minutes on an orbital shaker at room temperature. Membranes were incubated with goat anti-rabbit Dylight 680 conjugated secondary and/or goat anti-mouse Dylight 800 (Thermo-Scientific) conjugated secondary at 1:15000 in TBST for 30 minutes on an orbital shaker. Membranes were washed 3X in TBST for 5 minutes on an orbital shaker at room temperature before being imaged on LiCor scanner.

## 2.7 RNA INTERFERENCE AND SHORT HAIRPIN RNA

Transient knockdown of Amot was achieved using siRNA and control ON-TARGETplus (#1 D-001810-01-20, Dharmacon). For stable silencing, shRNA plasmids (Sigma - TRCN0000162009), or a shRNA sequence in pLKO.1 (Addgene) and Scramble shRNA control pLKO.1 (Plasmid #1864, Addgene) were transfected into 293T cells with packaging vectors by polyethylenimine (PEI) method. Lentiviral packaging and shRNA scramble control (1864) vectors were acquired from Addgene. shRNA plasmids directed against AmotL1 (Open Biosystems RHS3979-98825117) were also used to generate stably AmotL1 silenced cells.

## 2.8 RNA ISOLATION, CDNA GENERATION, AND QUANTITATIVE REAL TIME PCR

RNA Isolation: RNA was extracted for quantitative real time polymerase chain reaction (qRT-PCR) by the following 35mm-( Matrigel<sup>TM</sup> assay)/6cm<sup>2</sup>/10cm<sup>2</sup> (adherent cell) method: Media was aspirated, cells were washed with 1ml cold PBS. 1mL Tri-Reagent (Sigma Aldrich) was added directly to cells. Tri-reagent/cell solution was transferred to a 1.5mL (2mL for 35mm Matrigel<sup>TM</sup> assay) tube and incubated at room temperature for 5 minutes. 200µl chloroform was added followed by a 5 second vortex. Solution was allowed to incubate at room temperature for 5 minutes. Tubes were centrifuged at 4°C for 10 minutes at 20,817Xg. Aqueous phase containing RNA was transferred to new 1.5mL tube. Interphase (DNA) and organic phase (proteins, lipids) were discarded. RNA was precipitated with the addition of 500µl isopropanol. Tubes were vortexed subsequent to addition of isopropanol and allowed to incubate at room temperature for 5 minutes. Samples were centrifuged at 4°C for 10 minutes at 13K rpm. Supernatant was aspirated from tubes. RNA pellets were dislodged with 1mL cold 75% ethanol (EtOH). Samples were centrifuged at 4°C for 5 minutes at 10K rpm. EtOH was removed completely. RNA pellets were



allowed to dry for 5 minutes before resuspension in 22-50µl diethylpyrocarbonate (DEPC) treated water. RNA concentrations were determined by NanoDrop spectrophotometer (Nanodrop Technologies).

Generation of Complimentary DNA (cDNA): 2µg RNA were added to 2µl Oligo dT (Invitrogen) and 4µl dNTP (10mM) Mix (Invitrogen). Total volume was brought up to 24µl. Tubes were incubated for 5 minutes at 65°C. Tubes were spun down quickly and placed on ice. 9.5µl nuclease free water, 0.5µl RNase Out (Invitrogen), 4µl 10X reverse transcriptase (RT) buffer MulV (New England Biolabs), and 2µl in-house reverse transcriptase was added to tubes. Tubes were gently mixed with pipette tip then incubated at 50°C for 45 minutes. RT was inactivated by incubation at 85°C for 5 minutes. Samples were placed on ice.

Quantitative real-time PCR: All qRTPCR reactions were performed using a Realplex2 epGradient mastercycler (Eppendorf) with 2X SensiMix SYBR No-ROX Mastermix (BioLine) per manufacturer's instructions. Each 20µl sample contained 3µl ddH<sub>2</sub>O, 1µl sense oligo (2.5µM), 1µl anti-sense oligo (2.5µM), 10µl 2X SYBR Mastermix, and 5µl cDNA template.

## 2.9 THREE DIMENSION IrECM ASSAYS AND IMMUNOBLOT PROCEDURE

Matrigel™ Assay: MCF10A cells were seeded onto Matrigel™ for stereomicroscopic imaging, immunoblot analysis, and RNA extraction by the following method. 4 hours prior to seeding, 1mL aliquots of IrECM (Matrigel™) were placed on ice. Once depolymerization of Matrigel™ was apparent, MCF10A cells were trypsinized as noted in section 2.1. 150µl of Matrigel™ was spread per 35mm dish. Dishes were transferred to the tissue culture incubator and allowed to incubate for no more than 10 minutes. To generate “Feeder” media, to each 50mL of Assay media was added 2.5µl EGF (100µg/mL) and 1.25mL depolymerized Matrigel™. MCF10A cells were resuspended in 1mL MCF10A Assay media. Proper resuspension of cells

was essential to avoid the formation of cellular aggregates. Each 35mm dish received  $3 \times 10^5$  cells resuspended in 2mL Assay media. To avoid cellular aggregate formation, cells were diluted in MCF10A Assay media based on pellet size, typically between 10 and 14mLs total volume for a confluent 15cm dish. Cells were counted via hemocytometer. Total cells needed plus one assay were added to total volume of Assay media needed plus one assay e.g. if three 35mm dishes were needed ( $9 \times 10^5$  cells) a total of  $1.2 \times 10^6$  cells would be resuspended in 8mLs Assay media and 2mLs solution would be added per 35mm dish. Cells would be allowed to adhere overnight and Assay media would be replaced with Feeder media the following morning. Feeder media was replaced every four days as needed.

**Immunoblotting of acini:** Proteins were extracted from acini and resolved by the following method. Feeder media was aspirated from plates on ice. Acini were gently washed with 500 $\mu$ L cold PBS. Acini were lysed on Matrigel<sup>TM</sup> by addition of RIPA with inhibitors as described above. Acini were scraped into a cell slurry with RIPA and transferred to a 1.5mL tube. Solution was passed 3X through a 27G needle into a 10mL syringe, then transferred to ice for 10 minutes. Tubes were centrifuged at 4°C for 15 minutes at 14K rpm. Supernatant was removed and transferred to new tube. Protein concentrations were normalized via CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) which quantitatively measures lactate dehydrogenase (LDH). LDH is a cytosolic enzyme not found in Matrigel<sup>TM</sup>. The Cytotox assay is a coupled enzymatic assay which results in the conversion of tetrazolium salt (INT) into a red formazan product directly proportional to the number of cells lysed. Absorbance is read at 490nm on a 96 well plate reader. Subsequent steps are described in section 2.6.

## 2.10 TEAD REPORTER ASSAY

Cells were transfected 24 hours post passaging with 0.02µg TK-Renilla as a transfection control, in addition to indicated plasmid vectors. Luciferase was allowed to accumulate for 8 hours before cell lysis in passive lysis buffer per manufacturer's instructions (Promega). Samples were processed per manufacturer's instructions for Dual-Luciferase Reporter Assay System (Promega TM040). Samples were analyzed using a dual luminometer (BioSystems). All cells were transfected via PEI as described in section 2.5. All luciferase data represent the ratio of Firefly luciferase counts over control Renilla luciferase counts.

## 2.11. TRITIATED THYMIDINE INCORPORATION AND ACCUMULATION ASSAY

Cellular proliferation was measured as a function of [<sup>3</sup>H] thymidine incorporation. 5,000 cells were seeded into each well of a 96 well plate. Within 24 hours post passaging a 6-hour pulse of 1.0 µCi of [<sup>3</sup>H] thymidine was applied, cells were then automatically harvested. The level of [<sup>3</sup>H] thymidine incorporated into nucleic acids, isolated via filter binding, was determined as counts per minute (CPM).

## 2.12. NUCLEAR FRACTIONATION METHOD

To enrich the nuclear fraction of proteins for immunoblot analysis, the following method was used. Nuclear Fractionation Buffer A (NFB-A) (pH 7.9) was generated by adding 10mM HEPES, 1.5mM MgCl<sub>2</sub>, 10mM KCl, and 0.05% octylphenoxypolyethoxyethanol (IGEPAL). 6X10<sup>6</sup> control and treatment cells were seeded onto a 10cm<sup>2</sup> dish 24 hours before harvesting. Media was aspirated, and cells were washed with cold PBS. 500µL NFB-A, supplemented with

inhibitors as noted above, was added directly to cells. Cells were scraped into a 1.5mL tube and incubated on ice for 10 minutes. Cells were centrifuged at 3K rpm for 10 minutes at 4°C. Supernatant was removed and labeled cytosolic fraction. Cell pellet was resuspended in 1mL NFB-A. The tube was centrifuged at 956Xg for 1 min at 4°C. Supernatant was discarded and two more resuspensions were performed. Each pellet was then resuspended in 80-100µl RIPA. Cells were incubated on ice for 10 minutes before centrifugation at 20,817Xg for 10 min at 4°C. Cytosolic and nuclear fraction protein concentrations were determined with BCA as described above.

### 2.13. IMMUNOFLUORESCENCE MICROSCOPY

Confocal images were acquired on an Zeiss Axio ObserverZ1 as structured light via an Apotome and processed with Axiovision 4.7. Stereo images were acquired using a Nikon SMZ1500 microscope. Immunofluorescence was performed as described in (Heller et al., 2010) with the following antibody dilutions:  $\alpha$ -Par-3 1:800 (Zymed),  $\alpha$ -Amot 1:200,  $\alpha$ -PO4-ERK1/2 1:500,  $\alpha$ - ERK1/2 1:500,  $\alpha$ - Yap 1:500, and  $\alpha$ - Lats1 1:500. Normal and tumor mammary tissue sections were exposed to a photobleaching treatment with a high intensity blue (single wavelength at 450nm) light emitting diode (LED) semi-conductor light source for a duration of 24 hours prior to the de-paraffinization and slide processing. Sections were then immunolabelled with Amot anti-rabbit at a dilution of and  $\beta$ -catenin anti-mouse (Cell Signaling) at a dilution of 1:1000. Secondary antibodies included Alexa 546 and Alexa 488 (Invitrogen) both at a dilution of 1:500. Nuclei were stained with Hoechst. All labels were diluted in blocking buffer solution of (PBS/5%BSA/1%Saponin). The three fluorescent signals were excited with the Zeiss epifluorescent YFP, DsRed, and DAPI filter sets.

### **CHAPTER 3. THE ADAPTOR PROTEIN AMOT PROMOTES THE PROLIFERATION OF MAMMARY EPITHELIA VIA PROLONGED ACTIVATION OF ERK1/2**

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### 3.1. INTRODUCTION

The asymmetrical organization of mammary epithelial cells is a requirement for proper maintenance of cellular quiescence in developmental processes (Bissell et al., 2003). During mammary organogenesis, epithelial cells lining ducts are oriented with an apical domain facing the lumen and a basal domain which interacts with myoepithelial cells and stromal tissue (Itoh and Bissell, 2003). Apical polarity is maintained by the dynamic interactions between the Crb and Par polarity complexes (Shin et al., 2006). Several studies have demonstrated that disruption of these complexes facilitates growth mechanisms (Feigin and Muthuswamy, 2009; Rubin, 2008; Tanos and Rodriguez-Boulan, 2008). Polarity complexes maintain the stability of apical junctions through orientation of vectorial trafficking (Lene N. Nejsun, 2009). Of particular significance is the finding that both disruption of complex function and location mediates cell proliferation in mammary epithelia (Aranda et al., 2006; Huang and Muthuswamy, 2010; Joberty et al., 2000).

The adaptor protein Amot regulates apical polarity by controlling the spatial distribution of the Par and Crb complexes (Wells et al., 2006). The two characterized domains of Amot shed light on its dual functionalities. The C-termini of the 80kDa isoform of Amot, (Amot80) encodes a PDZ binding motif which directly interacts with the PDZ domain containing polarity proteins PatJ and Mupp1 which, in turn, scaffold Par and Crb proteins (Wells et al., 2006). The N-termini of Amot80 contains a more recently described Amot coiled-coil homology (ACCH) domain which binds membranes enriched in recycling endosomes (Heller et al., 2010). Together these two domains allow Amot to bind polarity complexes and redistribute them from apical junctions into recycling endosomal compartments (Heller et al., 2010). Thus, Amot80 induces the redistribution of apical polarity complexes, leading to disruption of apical junctions. Disruption of apical junctions is an early event in loss of cellular differentiation (Itoh and Bissell, 2003).

Several lines of evidence prompted this investigation of the role of Amot80 in mammary oncogenesis. First, Amot appears to coordinate polarity proteins during cell migration. For instance, Amot expression is upregulated in epithelial cells in the elongating trophoblast (Ross et al., 2009), and anterior visceral endoderm (Shimono and Behringer, 2003), and in endothelial cells undergoing angiogenesis (Shimono and Behringer, 2003). Second, Amot expression is significantly upregulated in response to inflammatory signaling subsequent to cutaneous wounding (Roy et al., 2008). These data suggest a link between inflammation and epithelial migration. Finally, Amot transcript levels correlate with breast tumor grade, and poor patient outcome (Jiang et al., 2006).

The following data describe a novel role for Amot80 in promoting prolonged Erk1/2 activation in mammary epithelial cells. Several cellular contexts were investigated to uncover the role of Amot80 in both initiation and promotion of aberrant growth in mammary epithelia. For instance, the growth rates of the hormone dependent luminal mammary cancer cell type MCF7 were significantly increased subsequent to Amot80 expression via Erk1/2 activation. Growth arrest and differentiation were overcome in the non-tumorigenic MCF10A cells grown as acini on Matrigel<sup>TM</sup> subsequent to Amot80 expression, and finally Amot expression in the triple negative mammary cell type MDA-MB-468 was shown to be required for growth.

## 3.2. RESULTS

### 3.2.1. Amot is Required for Prolonged Activation of Erk1/2 in HEK293T Cells.

The effect of MAPK (Erk1/2) activation is determined in large part by location (Murphy and Blenis, 2006). Erk1/2 activation at the plasma membrane results in transient activation of cytosolic processes whereas Erk1/2 activation on endosomes results in prolonged signaling linked to nuclear transcription of pro-growth genes (Brown and Sacks, 2009). Amot expression has

been shown to redirect apical complexes from junctional landmarks to endosomes (Heller et al., 2010) therefore suggesting a possible role in mediating prolonged MAPK activation.

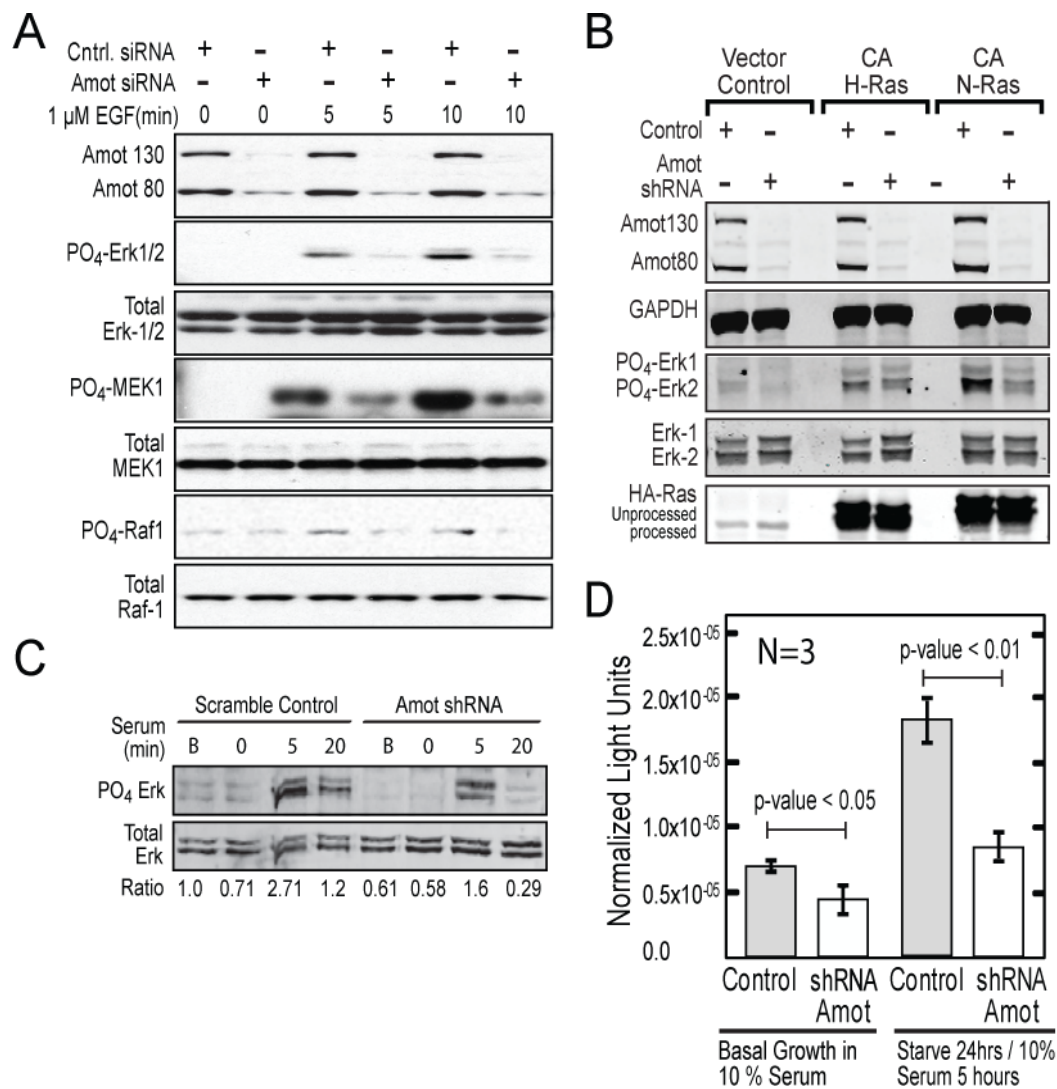
The involvement of Amot in mediating MAPK activation was first studied in HEK 293T cells given their high levels of endogenous Amot expression. 293T cells were assayed for changes in canonical MAPK pathway effectors Raf1 (340Y/341Y), Mek1 (217S/221S), and Erk1/2 (202T/204Y). siRNA mediated reduction in Amot expression was followed by EGF induction of MAPK effectors. The fractions of each of the phosphorylated effectors, when Amot expression was lacking, was observed to be diminished (Figure 3-1A).

Since Amot expression appears to be required for activation of MAPK effectors, the investigation next focused on the molecular points in the MAPK pathway where Amot was required. To address this, cells stably silenced for Amot expression were transfected with constitutively active H- and N-Ras. An approximately 40% reduction in Erk1/2 phosphorylation was observed in H-Ras transfected cells while an 90% reduction in Erk1/2 phosphorylation was observed in N-Ras transfected cells compared to control (Figure 3-1B). These data are consistent with Amot recognizing and regulating a specific membrane microdomain similar to those observed in N-Ras activation (Matallanas et al., 2003) and suggest a role for Amot in promoting Ras activation of Raf.

The observation that Amot is required for Erk1/2 activation, in addition to the known role for Amot in endosomal recognition, prompted the investigation of whether Amot was mediating prolonged or transient Erk1/2 activation. 293T cells stably silenced for Amot expression, and control cells, were serum starved for 24 hours prior to incubation with DMEM and 10% serum for 5 and 20 minutes. Cells which lacked Amot expression showed an approximate 75% reduction of Erk1/2 activation (Figure 3-1C). These findings suggest that Amot regulates the fraction of Erk1/2 involved in nuclear transcription of pro-growth genes. Activated Erk1/2 enters the nucleus where it phosphorylates the ternary complex transcription factor Ets-like gene 1 (Elk1) (Gille H, 1995), promoting serum response factor (SRF) binding and a transcriptionally



active complex (Brunet et al., 1999; Shaw and Saxton, 2003). To assess Erk1/2 nuclear activity, luciferase driven by an Elk1 promoter was measured under basal and serum stimulated conditions in 293T cells stably silenced for Amot expression and control. Under basal growth conditions loss of Amot expression resulted in a modest but significant ( $p < 0.05$ ) decrease in Elk1 reporter activity while cells which were lacking Amot expression showed a dramatic ( $p < 0.01$ ) inability to activate Elk1 in response to serum stimulation (Figure 3-1D).



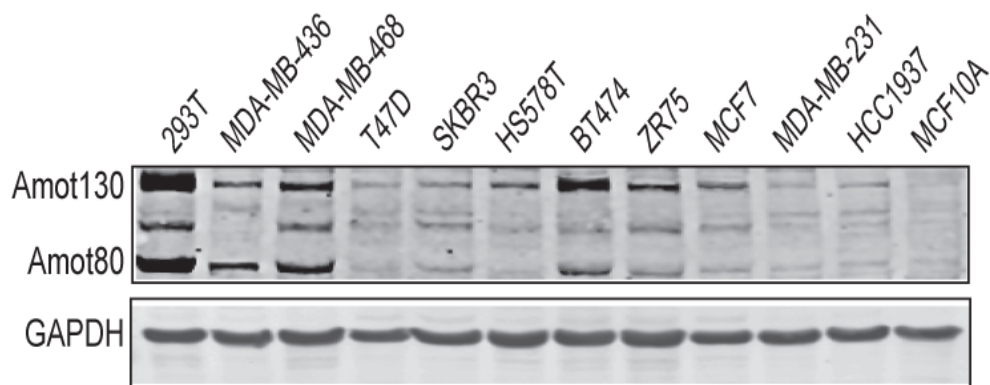
**Figure 3.1 Amot is Required for Prolonged Activation of Erk1/2.**

A. HEK 293T cells were serum starved for 24 hours and then stimulated with EGF for 5 and 10 minutes. Levels of phosphorylated and total Raf1, Mek1, and Erk1/2 as well as Amot were measured by immunoblot (Data generated by Zhang Han). B. Constitutively active (CA) mutants of H-and N-Ras were transiently expressed in HEK 293T cells stable infected with lentivirus encoding scramble control or shRNA against Amot. The levels of Amot, GAPDH, Ha-Ras, and phosphorylated and total Erk1/2 were measured by immunoblot. C. A bulk population of HEK293T cells stably silenced for Amot expression by infection with lentivirus were cultured in 10% serum (basal-B) or in DMEM alone and then treated with 10% serum for indicated time points. The levels of phosphorylated and total Erk1/2 were measured by immunoblot. The ratio of phosphorylated over total Erk1/2 is listed below. D. The normalized levels of luciferase expressed from an Elk1 reporter plasmid in control or Amot silenced cells are plotted as the mean of 3 independent samples. Assays were performed using cells cultured under basal conditions or serum starved for 24hours then serum added back for 4 hours. Error was computed as standard deviation of the mean. P-values are derived from unpaired 2-tailed student t-test (Ranahan et al., 2011).

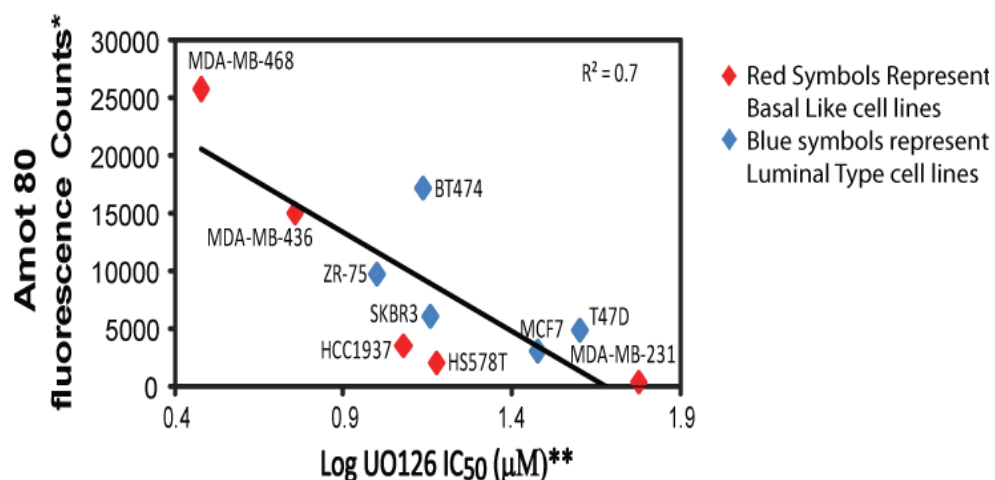
### 3.2.2. Comparative Expression of Amot in Human Mammary Cell Lines and Correlation of Amot Expression with Sensitivity to Growth Inhibition by U0126.

Ductal epithelial cells require Erk1/2 activation to proliferate during organogenesis and oncogenesis (Harari and Yarden, 2000; Hens and Wysolmerski, 2005). In an effort to better contextualize an Amot requirement for prolonged Erk1/2 activation in mammary epithelia, an assessment of relative levels of Amot expression was pursued across a panel of mammary cell lines. These cell lines represent a range of hormone and growth factor receptor statuses (Lacroix M, 2004). Amot80 and Amot130 were detected most highly in MDA-MB-468, 436 and BT474 cells. MCF7, HS578T, and ZR75 displayed moderate Amot130 levels and low Amot80 levels while SKBR3 had equally low Amot80/Amot130 levels. T47D, MDAMB-231, HCC1937, and MCF10A cells showed the lowest Amot expression (Figure 3-2A). Importantly, the relative expression of Amot was found to correlate (Coefficient of Determination  $R^2$  value of 0.7) with the known susceptibility of these cells to be growth inhibited by treatment with MEK inhibitor U0126 (Mirzoeva et al., 2009) (Figure 3-2B).

A



B



**Figure 3-2 Correlation of Amot80 Protein Levels with Sensitivity of Breast Cells to Growth Inhibition by UO126.**

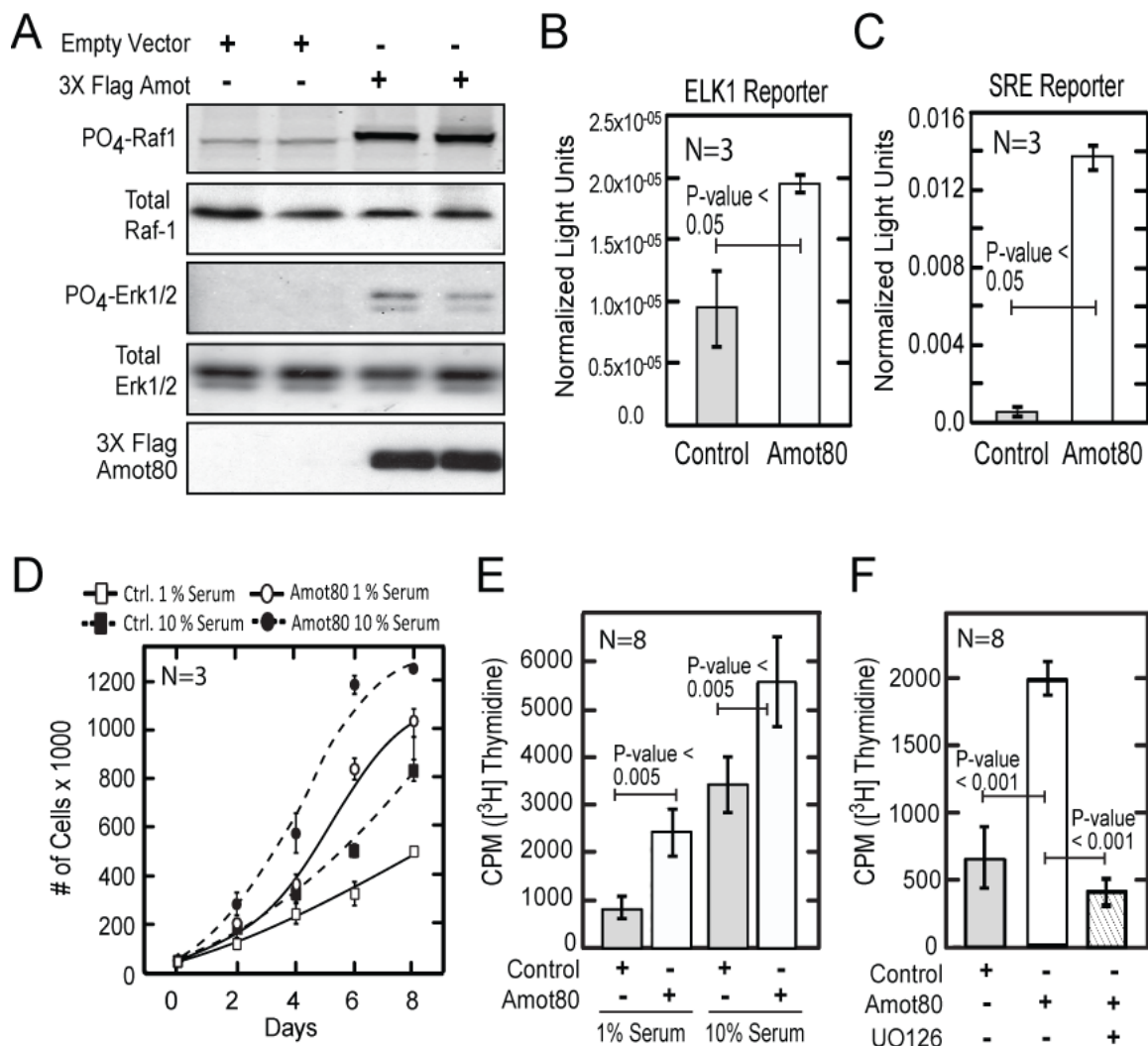
A. Mammary cells were grown in appropriate growth media until approximately 80 % confluent. Cells were then scraped in PBS at 4°C, centrifuged at 1000 x G for 1 minute at 4°C. Cells were then resuspended in PLC lysis buffer with protease and phosphatase inhibitors and immediately flash frozen on dry ice. The post-nuclear extract was then boiled in sample buffer and analyzed by immunoblot. B. The fluorescent intensities of the Amot80 band shown in A was measured using a LiCor Odyssey and plotted versus the IC<sub>50</sub> values, as calculated by (Mirzoeva et al., 2009), for growth inhibition by UO126 (Ranahan et al., 2011).

### 3.2.3. Amot80 Expression Increases Erk1/2-Dependent Signaling to Enhance the Rate of Proliferation of MCF7 Cells.

Cell lines with high levels of Amot80 expression correlate with sensitivity to U0126 inhibition of Erk1/2, and require Amot expression to mediate MAPK signaling. These data suggest a role for Amot80 in promoting prolonged Erk1/2 activation. To test this directly, Amot80 was stably expressed in MCF7 cells. Unlike many tumorigenic mammary cell types, MCF7 cells display unusually developed apical junctions as evidenced by the localization of occludin, a tight junction transmembrane protein, to cell-cell contacts (Martin and Jiang, 2009). Amot80 expression in MCF7 cells is therefore expected to disrupt apical polarity. In addition, MCF7 cells represent an estrogen dependent tumor type which can switch to Erk1/2 signaling for growth following anti-estrogen treatment (Gee et al., 2005). It is unclear whether loss of apical polarity is correlative or causal in recurrence of anti-hormone treated tumors. Thus elucidation of the mechanisms whereby tumor cells become resistant to hormone therapy would likely provide novel targets for resistant tumor types.

The effects of heterologous expression of Amot80 on MAPK signaling and the mitotic rate in MCF7 cells were investigated. Flag-tagged Amot80 and control Flag were stably expressed via retroviral mediated gene delivery. Flag-Amot80 expressing cells showed enhanced basal levels of Raf1 and Erk1/2 phosphorylation compared to control (Figure 3-3A). Consistent with prolonged Erk1/2 activity being associated with transcriptional activation, both Elk1 and SRE luciferase reporters demonstrated significantly higher (over 2-fold and 20-fold respectively) luciferase activity in cells stably expressing Amot80 compared to control (Figure 3-3B,C). Given that Amot80 expression increased basal Erk1/2 phosphorylation and transcriptional activity, its impact on cellular proliferation was measured. Over the course of 8 days, MCF7 cells stably expressing cerulean fluorescent protein (CFP) alone or Amot80-CFP were counted. Cells expressing Amot80 accumulated 2 to 3 fold faster under 1% serum and approximately 70% faster in 10% serum versus control cells (Figure 3-3D). Consistent with these observations, MCF7 cells

expressing Amot80-CFP incorporated 2 to 3 fold more [<sup>3</sup>H]thymidine into nucleic acids over a six hour period compared to control (Figure 3-3E). The Amot80 induction of [<sup>3</sup>H] thymidine incorporation was inhibited by treatment with U0126 demonstrating that Amot80 expression enhances cell proliferation in an Erk1/2 dependent manner (Figure 3-3F).



**Figure 3-3 Amot Induces ERK1/2-Dependent Proliferation of MCF7 Cells.**

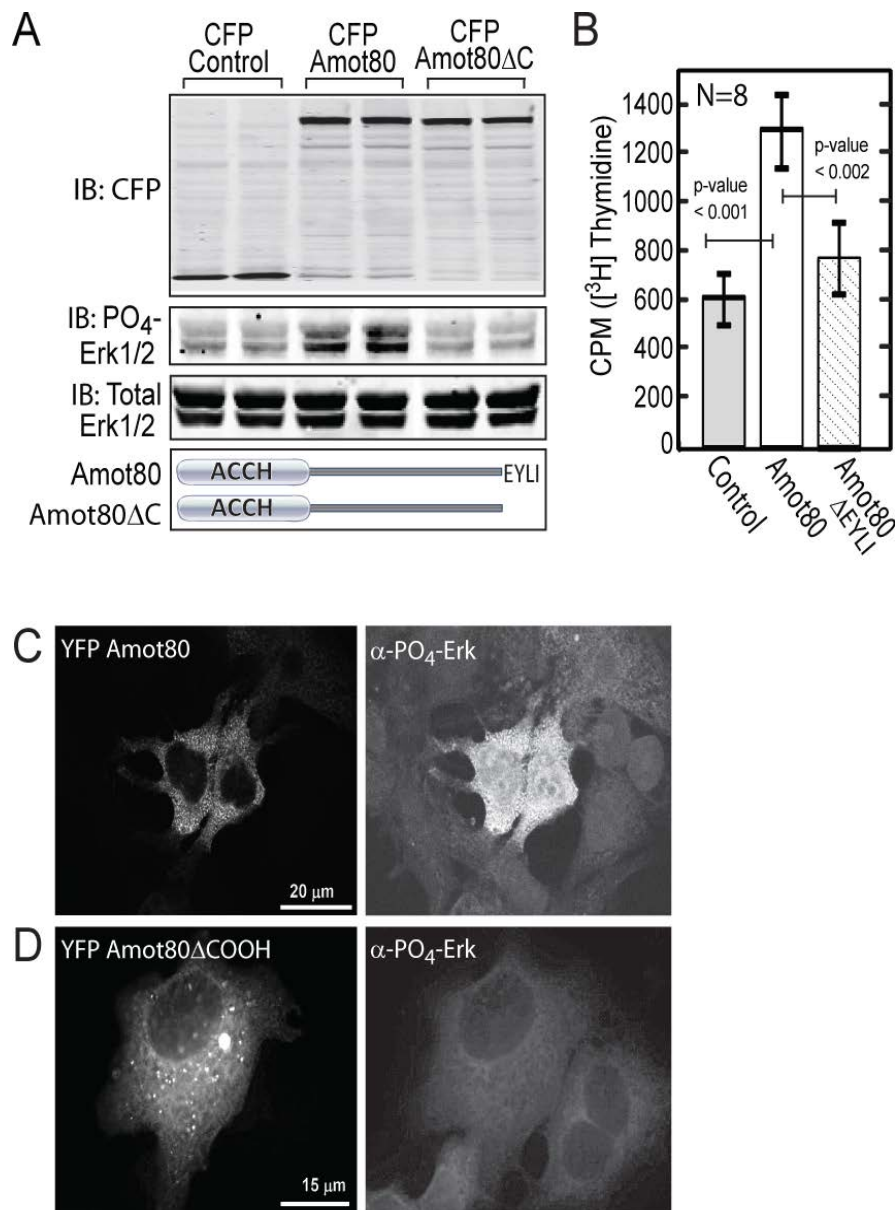
A. The relative levels of Flag-tagged Amot80, phospho- and total Raf1, and ERK1/2 were measured in lysates from MCF7 cells stably expressing control or Flag-tagged Amot80 (Data generated by Zhang Han). B. Plot of the normalized luciferase activities of MCF7 cells expressing CFP or CFP-tagged Amot80 and the ELK-1 reporter construct. C. Plot of the normalized luciferase activities of MCF7 cells expressing CFP or CFP-tagged Amot80 and the SRE reporter construct. D. The mean number of MCF7 cells at the indicated days from 3 independent experiments are plotted where cells grown in 1% serum (solid lines) and expressing CFP (□) or CFP-tagged Amot80 (○) as well as cells grown in 10% serum (dashed lines) and expressing control CFP (■) or CFP-tagged Amot80 (●) are depicted (Data generated by Whitney Smith-Kinnaman). E. The mean levels of [<sup>3</sup>H] thymidine incorporated into nucleic acids from 8 replicates of MCF7 cells expressing control (grey bars) or Amot80 (white bars) (Data generated in collaboration with Sarah Nabinger). F. MCF7 cells expressing the indicated constructs were grown in 1% serum and 10 mmol/L UO126 or DMSO were assayed as described in E (Data generated in collaboration with Sarah Nabinger). Error represents the standard deviation of the mean. P values are from unpaired/2-tailed student t tests (Ranahan et al., 2011).

#### 3.2.4. Amot80 Requires an Intact PDZ Binding Domain to Activate Erk1/2 and Promote Cell Proliferation.

Previous reports have demonstrated that Amot80 requires its C-terminal PDZ binding domain to coordinate chemotactic migration and to disrupt apical junctions (Levchenko et al., 2003; Wells et al., 2006). Amot80 expression has been shown to induce disruption of apical junctional integrity by internalizing members of the Crb and Par complexes. This re-orientes apical trafficking patterns to recycling endosomal compartments (Heller et al., 2010; Wells et al., 2006). These studies prompted the investigation into whether Amot80 also required an intact PDZ domain to promote prolonged Erk1/2 phosphorylation and cell growth. Control CFP along with Amot80-CFP and an Amot80-CFP construct lacking the four C-terminal amino acid residues (Amot80 $\Delta$ C) were stably expressed via retrovirus in MCF7 cells.

Lysates from these cells were subjected first to western immunoblot analysis where Amot80 expressing cells, but not control or Amot80 $\Delta$ C expressing cells, showed enhanced basal levels of Erk1/2 phosphorylation (Figure 3-4A). Next, these cells were subjected to [ $^3$ H] thymidine incorporation analysis where Amot80 expressing cells, but not control or Amot80 $\Delta$ C expressing cells, displayed a 2-fold increase in [ $^3$ H] thymidine incorporation (Figure 3-4B). Finally, cells expressing YFP, Amot80-YFP, or Amot80 $\Delta$ C-YFP were subjected to immunofluorescence-based analysis. Cells were fixed and immunostained for phosphorylated and total Erk1/2. Cells expressing Amot80, but not Amot80 $\Delta$ C, showed increased staining of phosphorylated Erk1/2 (Figure 3-4C,D). Given the previous reports indicating Amot80 redistributes polarity proteins to recycling endosomes (Heller et al., 2010; Wells et al., 2006), it is likely these are the same subcellular locales where Amot80 is promoting Erk1/2 activation.



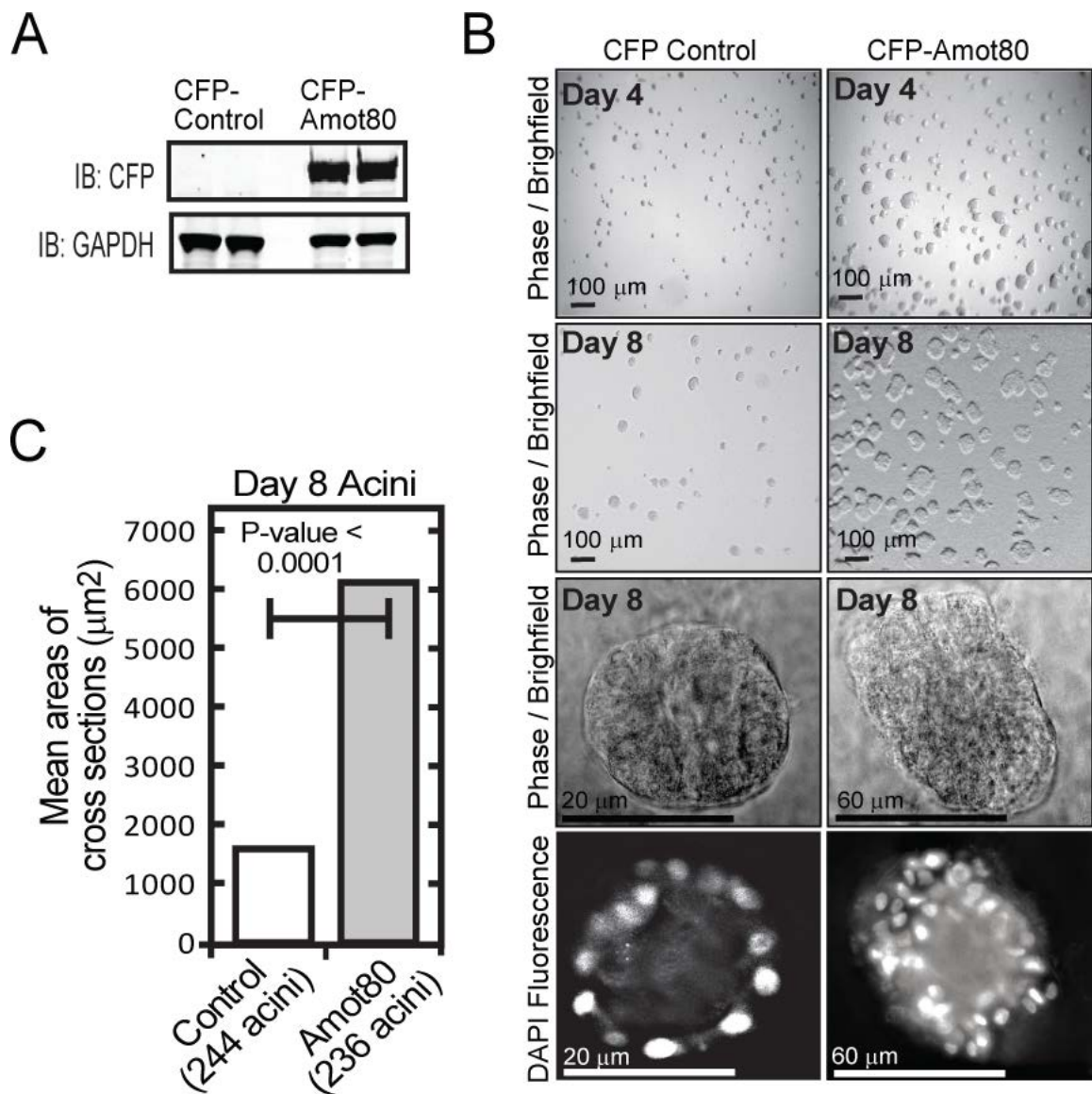


**Figure 3-4 Amot80 Requires an Intact C-Terminus to Activate ERK1/2 and Cell Proliferation.**

A. Lysates from MCF7 cells infected with retrovirus encoding CFP, CFP-tagged Amot80, or CFP-tagged Amot80ΔC were immunoblotted for CFP, phospho-ERK1/2, and total ERK1/2. A cartoon representation of Amot80 and Amot80ΔC proteins is depicted in the bottom. B. The levels of [<sup>3</sup>H] thymidine incorporated into nucleic acids by MCF7 cells grown in 1% serum and expressing CFP control (grey bars), CFP-tagged Amot80 (white bars), or CFP-Amot80ΔC (hatched bars) (Data generated in collaboration with Sarah Nabinger). C. A confocal image of fluorescence in MCF7 cells expressing YFP-tagged Amot80 (left) and immunostained for phospho-ERK1/2 (right). D. Confocal image of YFP-tagged Amot80ΔC (left) and an immunostain of phospho-ERK1/2 (right) in MCF7 cells (Ranahan et al., 2011).

### 3.2.5. Expression of Amot80 Induces Non-Polarized Growth of MCF10A Cells in Matrigel<sup>TM</sup>.

Mammary epithelia, whether tumorigenic or not, remains poorly differentiated when passaged on traditional 2D plastic substrata. This discrepancy is due to 2D plastic substrata lacking the extracellular cues which mediate gene expression and regulate differentiation *in vivo* and in 3D IrECM (Matrigel<sup>TM</sup>) environments (Debnath et al., 2003; LaBarge et al., 2007). MCF10A cells are a spontaneously immortalized, non-tumorigenic, mammary cell line which forms monolayers on plastic in a manner similar to MCF7 cells. MCF10A cells, however, when seeded onto Matrigel<sup>TM</sup>, form proliferation arrested acini with hollow lumens encompassed by a monolayer of polarized cells. These acini are functionally very similar to ductal epithelial tissues *in vivo* (Debnath and Brugge, 2005; Debnath et al., 2003; Kenny et al., 2007; Muthuswamy et al., 2001). To examine the impact of Amot80 expression on non-tumorigenic epithelial cells grown in a growth inhibitory environment, MCF10A cells stably expressing Amot80-CFP or CFP alone were grown in Matrigel<sup>TM</sup> (Figure 3-5A). Within 4 days, MCF10A cells expressing Amot80 developed into colonies which were visibly larger than control cells. By 8 days post entry into Matrigel<sup>TM</sup> Amot80 expressing MCF10A cells were over 4-fold larger in size (Figure 3-5B). Confocal imaging of 4', 6' diamidino-2' phenylindole (DAPI)-stained nuclei indicated that acini formed by Amot80 expressing MCF10A cells contained a greater number of cells and lacked a hollow lumen (Figure 3-5B bottom panel), consistent with defects observed in mammary ducts unable to down regulate Erk1/2 signaling (Turley et al., 2008).



**Figure 3-5 Heterologous Expression of Amot80 Promotes the Poorly Differentiated Outgrowth of MCF10A Cells Cultured in Matrigel™.**

A. The protein levels of CFP, CFP-tagged Amot80, and GAPDH in the indicated MCF10A lines were measured by immunoblot. B. Bright field stereo images of the indicated cells after 4 days (top row) and 8 days (second row) of growth in Matrigel. A high resolution image of cells after 8 days was imaged by bright field (third row) and a reconstructed z-stack of confocal images of nuclei stained with DAPI from the cells depicted above (fourth row). C. The mean cross-sectional area ( $\text{mm}^2$ ) of acini from 8 filters per condition was plotted. P value represents unpaired/2-tailed t test assuming unequal variance (Ranahan et al., 2011).

### 3.2.6. SKBR3 cells require Amot expression for Erk1/2 but not AKT dependent growth.

Mammary cancers may activate disparate signaling pathways based on availability of growth factors and in response to therapeutic inhibitors. However, the mechanisms involved in these conversions remain unclear (Citri and Yarden, 2006; Sergina et al., 2007).

Microenvironmental cues have also been shown to contribute to such signaling adaptations.

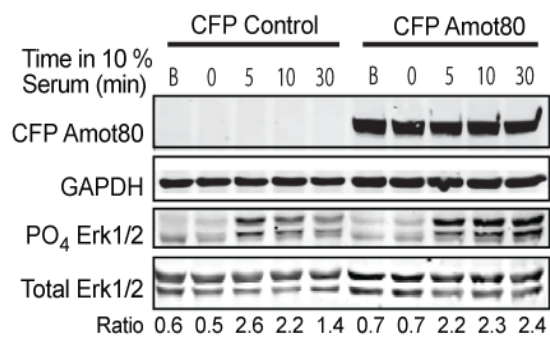
Upon transfer to Matrigel<sup>TM</sup>, SKBR3 cells upregulate ErbB2 expression and switch from AKT to Erk1/2 dependent growth signaling (Pickl and Ries, 2008; Weigelt et al., 2010). This phenomenon was exploited to determine whether Amot80 functions to promote growth selectively through Erk1/2. The effects of both heterologous expression and silencing of Amot in SKBR3 cells grown on plastic and in Matrigel<sup>TM</sup> were investigated.

Amot80-CFP and CFP alone were stably expressed in SKBR3 cells cultured on plastic and stimulated with 10% serum over a time course encompassing both transient and prolonged Erk1/2 activation. Relative levels of CFP, phosphorylated and total Erk1/2, and GAPDH were determined via western immunoblot analysis. Unlike MCF7 cells, SKBR3 cells expressing Amot80 did not show increased basal levels of Erk1/2 phosphorylation; they did, however, have increased levels of phosphorylated Erk1/2 at 30 minutes (Figure 3-6A) and modest but significant increase ( $p < 0.01$ ) in cellular accumulation as well as increased thymidine incorporation into DNA in a U0126 dependent manner (Figure 3-6B,C). Lentiviral mediated stable silencing of Amot had no marked effect on basal Erk1/2 phosphorylation (Figure 3-6D), growth rates, or thymidine incorporation into DNA (Figure 3-6C lanes 5-6). Thus SKBR3 cells grown on plastic showed a modest growth increase and enhancement of prolonged Erk1/2 phosphorylation, whereas loss of Amot expression had no discernible impact on basal growth.

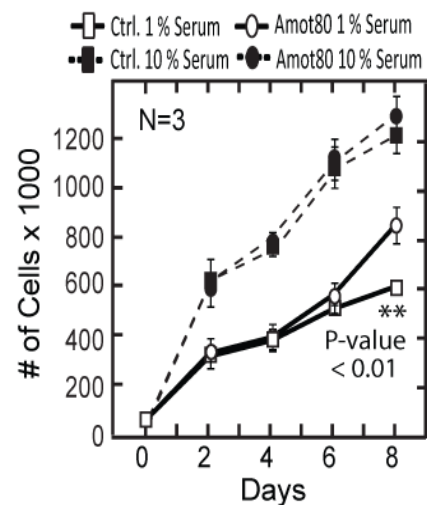
The requirement for Amot in SKBR3 cells under Erk1/2 dependent growth conditions (Matrigel<sup>TM</sup>) was next defined. SKBR3 cells grown on Matrigel<sup>TM</sup> and treated with U0126, but not control DMSO treated cells, were strongly growth inhibited as has been previously reported (Pickl and Ries, 2008; Weigelt et al., 2010). SKBR3 cells stably silenced for Amot expression

which grew normally on plastic displayed a complete loss of growth when transferred to Matrigel<sup>TM</sup> (Figure 3-6D-F). These data strongly suggest Amot acts specifically to mediate growth in mammary epithelial cells through Erk1/2 signaling.

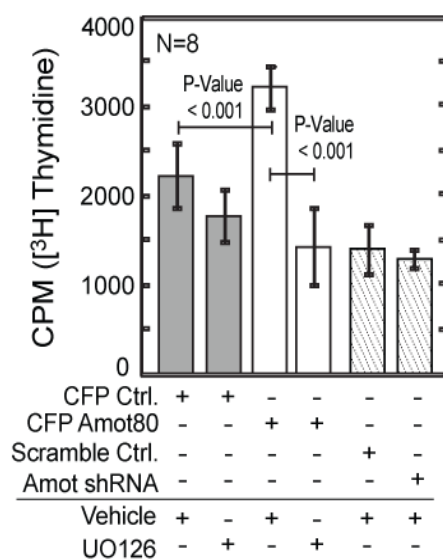
A



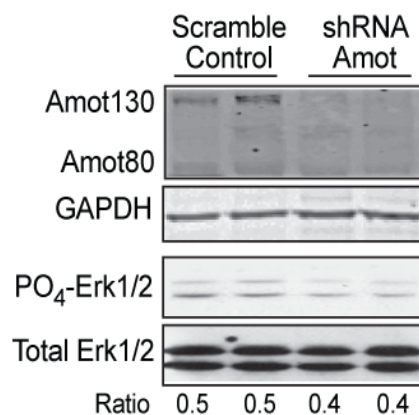
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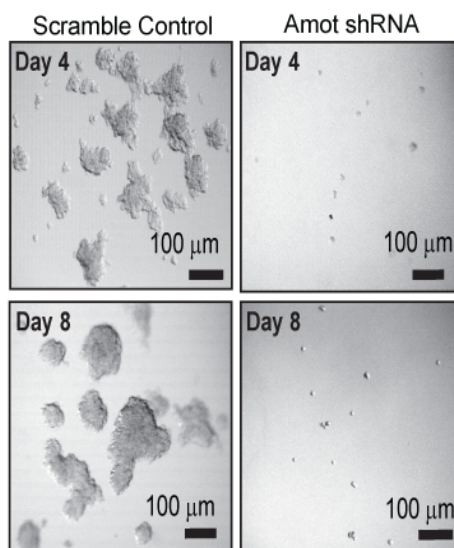
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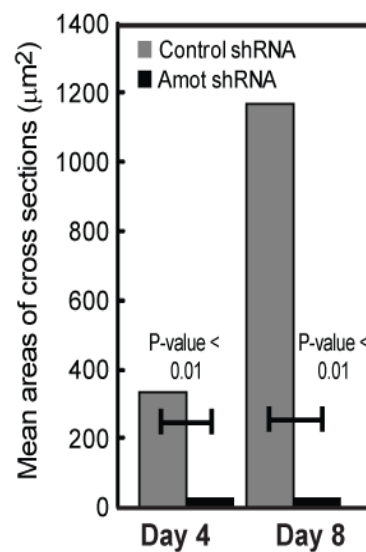
D



E



F



**Figure 3-6 Amot is Essential for ERK-Dependent Proliferation of SKBR3 Cells.**

A. Control and Amot80 expressing SKBR3 cells were stimulated with serum before the levels of GFP, GAPDH, and phospho- and total ERK1/2 were measured by immunoblot. The ratio of fluorescence intensities of phospho- and total ERK1/2 are given below. B. After the indicated number of days of growth cells were counted from 3 independent experiments. The mean number cells grown in 1% serum (solid line) control vector (□) or CFP-tagged AMOT80 (○) or 10% serum (dashed line) control vector (■) or CFP-tagged Amot80 (●) are plotted (Data generated in collaboration with Whitney Smith-Kinnaman). C. SKBR3 cells from A and B were plated in 10% serum with vehicle or U0126 for 24 hours before [<sup>3</sup>H] thymidine incorporation was measured. The mean from 8 replicates was plotted. (Data generated in collaboration with Sarah Nabinger). D. Bulk populations of SKBR3 cells infected with lentivirus-encoding scramble control or Amot shRNA were immunoblotted for Amot, GAPDH, and phospho- and total ERK1/2 (Data generated in collaboration with Whitney Smith-Kinnaman). E. Bright field stereo images of SKBR3 cells from B grown in Matrigel for 4 and 8 days. F. The mean cross-sectional areas (mm<sup>2</sup>) of colonies described in E from 8 filters per condition were plotted. P values were derived from unpaired/2-tailed t tests assuming unequal variance. (\*\* P < 0.01) (Ranahan et al., 2011).

### 3.3. DISCUSSION

#### 3.3.1. A Novel Role for Amot80 in Erk1/2 Dependent Proliferation of Mammary Epithelial Cells.

Several lines of evidence point to the importance of MAPK signaling during breast cancer progression, however, the mechanisms involved are varied and poorly understood. Nearly one third of all breast cancers require Erk1/2 signaling to grow (Navolanic et al., 2003) (Slamon DJ, 1989). Those cancers which do not initially require Erk1/2 activity to grow may upregulate MAPK signaling subsequent to chemotherapy and hormonal treatment to acquire therapeutic resistance (Arpino et al., 2005) (Dent et al., 2007). Of special concern is the “triple negative” tumor type, which is able to phosphorylate Erk1/2 without the expression of canonical upstream activators and is particularly resistant to current treatments (Bayraktar and Glück, 2013; Foulkes et al., 2010; Hudis and Gianni, 2011; Shastri and Yardley, 2013). Elucidation of the mechanisms promoting prolonged Erk1/2 activation in mammary epithelia is therefore significant to breast cancer therapy.

In this study a novel role for Amot80 is described in mediating prolonged Erk1/2 activation in mammary epithelial cells. Amot80 requires the ability to bind polarity proteins to mediate Erk1/2 phosphorylation, likely at the level of Raf1 activation by Ras. The observation that Amot80 expression promotes proliferation of MCF7 cells, coupled with Amot80 expression disrupting polarized acini formation, suggests that Amot80 coordinates loss of polarity with growth. Consistent with this, loss of Amot expression reduces Erk1/2 dependent growth in SKBR3 cells.



### 3.3.2. Proposed Mechanism of Amot80 Induced Growth Rate Increase in Mammary Epithelia.

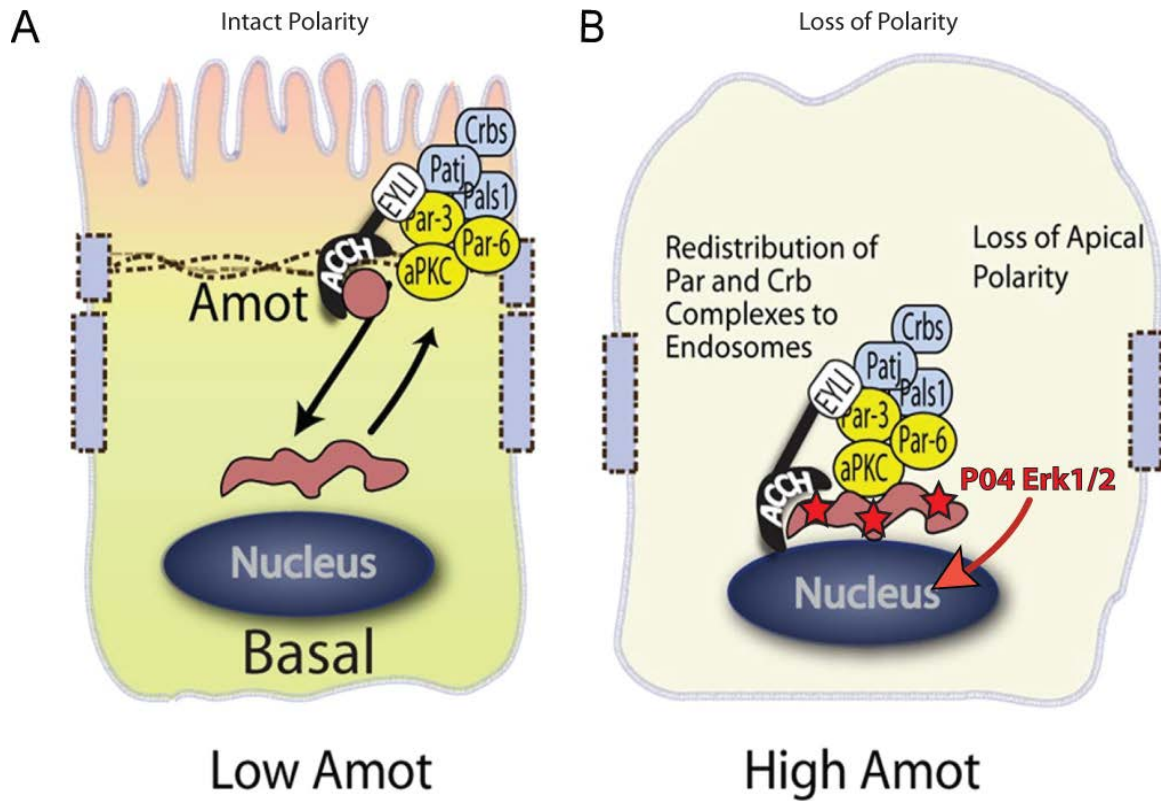
It is likely that Amot80 expression promotes growth through prolonged Erk1/2 phosphorylation. Amot80 expression enhanced basal levels of phosphorylated Erk1/2 and Raf1 (Figure 3-3A). Loss of Amot expression resulted in diminished ability of cells to phosphorylate Raf1, Mek1, and Erk1/2 in response to EGF or serum, suggesting an Amot requirement for MAPK activation at the Ras-Raf1 level (Figure 3-1A). The ability of Amot80 to increase mitotic rates of mammary epithelia (Figure 3-4B) is likely due to its preferential effects on basal or prolonged phosphorylation of Erk1/2, which has been linked to activation of ternary complex transcription factors driving proliferation (Brunet et al., 1999). This is directly supported by the observation that Amot80 expression activates both Elk1 and SRF transcription concomitant with increasing growth rates in MCF7 cells (Figure 3-3B, C).

It is probable that the recycling endosomes to which Amot80 redirects polarity proteins (Heller et al., 2010) are the same population of recycling endosomes involved in sustained Erk1/2 phosphorylation (Casar et al., 2009). In epithelial tissues Amot80 is known to recognize a juxtanuclear recycling endosomal compartment (Heller et al., 2010) while Amot80 and phosphorylated Erk1/2 co-distribute at intracellular compartments. The strong requirement of Amot downstream of N-Ras, which also binds cholesterol-rich vesicular membranes (Matallanas et al., 2003), is also consistent with this suggestion. Finally, Ras activation of Raf only results in a multi-hour pro-growth signal at endosomal compartments (Casar et al., 2009; Chiu et al., 2002). Taken together these data point to Amot80 coupling both polarity proteins and Erk1/2 into a slow trafficking pattern which extends Erk1/2 phosphorylation and prevents reestablishment of apical polarity.

### 3.3.3. Amot80 as a Diagnostic Tool in Breast Cancer Treatment.

While polarity protein complexes, such as Par and Crb, have known roles in mediating both the establishment of polarity and promoting growth (Lu and Bilder, 2005; Nolan et al., 2008), the causal factors governing the switch between these roles are still unclear (Huber et al., 2005; Miyoshi and Takai, 2005). As a general rule, the establishment and maintenance of epithelial polarity requires the correct localization of polarity constituents (Handler, 1989). As is plainly the case in migrating cells, change of polarity complex location results in change of function. The Par complex is apically sequestered and maintains junctional integrity in polarized cells, however, in mesenchymal cells the Par complex is at the leading edge coordinating chemotactic migration (Etienne-Manneville, 2008). It is therefore likely that Amot80 induced redistribution of polarity proteins to intracellular compartments is part of a larger signaling system coordinating loss of polarity with growth (Figure 3-7). The presence of Amot80, given its ability to dominantly disrupt apical polarity and promote growth, may serve as an indicator of polarity and growth status.

MCF7 cells represent a luminal, estrogen receptor positive (ER+), mammary tumor type. The effect of Amot80 expression in these cells reflects aspects of anti-estrogen resistance in luminal-type breast carcinomas. ER+ tumors often become resistant to anti-estrogen therapy by upregulating Erk1/2 dependent growth (Frogne et al., 2009). Amot80 expression may be one way luminal type cancers can escape estrogen dependence. Amot expression levels may also be an independent indicator of breast cancers that use Erk1/2 for growth. Amot expression levels correlate with high tumor grade and metastatic risk in breast cancer (Jiang et al., 2006) although, until this report, no causal role for Amot in breast cancer progression has been reported.



**Figure 3-7 Proposed Model of Amot80 Coordinated Loss of Polarity with Prolonged Erk1/2 Activation.**

A. Polarized epithelial cells maintain apical junctional integrity by dynamic interaction between Par and Crb complexes. Asymmetrical distribution of proteins and receptors prevents promotion of cell cycle. B. Under high Amot80 expression apical polarity complexes are internalized as junctional integrity is compromised. Previously sequestered receptor may now interact with agonists to promote growth. Phosphorylated Erk1/2 co-distributes with Amot80 on recycling endosomes prolonging Erk1/2 nuclear activation of transcription complexes driving pro-growth gene expression. Red arrows/star indicate novel finding while black arrows indicate previously identified pathways.

**CHAPTER 4. THE 80KDA ISOFORM OF AMOT IS A NOVEL ACTIVATOR OF THE  
ONCOGENE YAP**

#### 4.1. INTRODUCTION

The growth inhibitory effects of cell polarity in epithelia have long been appreciated (Feigin and Muthuswamy, 2009; Handler, 1989; Royer and Lu, 2011; Shin et al., 2006). Only recently, however, have the intracellular mechanisms underlying contact-mediated inhibition of growth begun to be understood (Boggiano and Fehon, 2012; Huang and Muthuswamy, 2010; Lelièvre, 2010). Subsequent to cells establishing contact, adherens junctions form providing a cellular landmark for polarity complexes such as the Par complex. As polarity is established, such complexes promote junctional stability while negatively regulating de-differentiation pathways like the  $\beta$ -catenin pathway (Lene N. Nejsun, 2009). Sequestration of the transcriptional coactivator  $\beta$ -catenin at adherens junctions is essential for maintenance of cellular quiescence.  $\beta$ -catenin that is not sequestered at apical junctions or degraded in the cytosol becomes available to translocate to the nucleus. Nuclear  $\beta$ -catenin binds T cell factor/lymphoid enhanced factor (TCF/LEF) transcription factors and activates expression of genes involved in de-differentiation and growth (Kraus et al., 1994; MacDonald et al., 2009).  $\beta$ -catenin regulation is therefore a prime example of one way in which epithelial cells translate cell-cell contact into growth inhibition.

In addition to  $\beta$ -catenin, the tight junction associated protein zona-occludin 1 (ZO-1) interacts with ZO-1-associated nucleic acid binding protein (ZONAB) to regulate gene expression. At low density ZO1 and ZONAB are located in the nucleus where they upregulate the mammary oncogene ErbB2. At high density ZO1 and ZONAB are localized to tight junctions where they maintain junctional integrity (Balda et al., 2003; Balda and Matter, 2000).

These examples illustrate how epithelial cells use tight and adherens junction proteins to maintain junctional stability and, through their disruption, to promote de-differentiation and growth. Location appears to be the final determinant of function. For example,  $\beta$ -catenin at

adherens junctions and ZO1 at tight junctions act to maintain polarity and inhibit growth. These same proteins in the nucleus have transcription functions that actively drive growth and de-differentiation.

The transcriptional coactivator Yes-associated protein (Yap) is the newest member of this group of apical-junction associated proteins to be described (Huang et al., 2005; Komuro et al., 2003). Like  $\beta$ -catenin, Yap is sequestered to apical junctions in cells at high density and localized in the nucleus in actively dividing cells (Zhao et al., 2007). Yap that is not sequestered at junctions or degraded in the cytosol is available for nuclear import where it complexes with TEA-domain containing (TEAD) transcription factors and drives expression of genes involved in growth and de-differentiation (Zhao et al., 2009; Zhao et al., 2010b; Zhao et al., 2008b). This likely underlies why Yap dominantly regulates organ size throughout metazoans (Dong et al., 2007) and is genetically amplified in breast cancers (Overholtzer et al., 2006). Consistent with Yap localizing to both tight and adherens junctions, it is regulated by several apical junction associated polarity proteins (Oka et al., 2010; Oka et al., 2012; Schlegelmilch et al., 2011; Silvis et al., 2011; Zhao et al., 2011a). Therefore, not surprisingly, Yap activity is inhibited by establishment of polarity (Zhao et al., 2007). Subsequent to the establishment of cell-cell contacts, Hippo pathway protein kinases Mst1/2 are activated, which in turn phosphorylate Lats1/2. Lats1/2 protein kinases phosphorylate Yap on serine 127 (Hao et al., 2008; Oka et al., 2008; Zhao et al., 2007). This phosphorylation event has been linked to cytosolic sequestration and degradation of Yap (Zhao et al., 2010b). Several non-Hippo pathway mechanisms of Yap regulation have also been demonstrated including models of cytoskeletal and junctional sequestration (Chan et al., 2011; Dupont et al., 2011; Yi et al., 2011). Given the universality of this pathway, and its dominant effects on organ size, the complexity of Yap regulation seems fitting. As the search for dominant regulators of Yap in epithelia has continued, the Amot family of polarity adaptors has emerged as central players in these events.

Recent studies have demonstrated that Amot family members Amot130, L1, and L2 are all involved in negative regulation of Yap through canonical Hippo signaling and via sequestration (Chan et al., 2011; Oka et al., 2012; Paramasivam et al., 2011; Varelas et al., 2010; Wang et al., 2011; Zhao et al., 2011a). Amot family members, with the exception of Amot80, have conserved N-terminal PPXY motifs which mediate binding to WW domains in Yap (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011a). Since Amot80 is N-terminally truncated, direct interactions with Yap is not predicted and this likely explains why the role of Amot80 in Yap regulation has not been examined.

The following studies describe a novel role for Amot80 in promoting Yap pro-growth nuclear activity. Effects of Amot80 expression on Yap localization, S127 phosphorylation, and nuclear activity were investigated in both non-tumorigenic and transformed mammary cell lines. A requirement of Amot expression for Yap activity was investigated using both traditional 2D plastic substrata as well as 3D Matrigel<sup>TM</sup> (i.e. polarizing and growth inhibitory conditions). Given that previous reports have described Amot family members as negative regulators of Yap, the relative contributions of Amot130 and AmotL1, which directly bind Amot80, were also investigated. Amot family members are found to be differentially regulated during mammary acini development. These data were exploited to uncover the differential requirements of Amot family members for Yap activity and growth. While Amot80 expression results in increased Yap activity and unorganized overgrowth of acini, expression of Amot130 and AmotL1 results in growth inhibition. Amot expression is shown to be required for Yap activity both during acini formation and in cells grown on plastic. Loss of AmotL1, however, results in increased Yap activity and failure of acini to arrest growth.

## 4.2. RESULTS

### 4.2.1. Expression of Amot80 Promotes and is Required for Yap Transcriptional Activity.

Previous reports have demonstrated that expression of Amot130, AmotL1, and AmotL2 expression results in decreased Yap activity in the nucleus (Chan et al., 2011; Oka et al., 2012; Paramasivam et al., 2011; Varelas et al., 2010; Wang et al., 2011; Zhao et al., 2011a). Many of these reports began with characterizations of Yap binding partners and their respective interactions where WW domains in Yap were discovered to bind PPXY motifs in the N-terminal regions of Amot family members (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011a). Since Amot80 is N-terminally truncated, it lacks the PPXY motif and therefore was not observed to interact with Yap. Consequently, all focus was on understanding the effects of full length Amot130 and the other family members on regulation of Yap activity. To date there have been no reported investigations into Amot80 regulation of Yap activity. Since expression of Amot130, L1, and L2 was shown to negatively regulate Yap this investigation began with heterologous expression of Amot80. The mammary cancer cell line MDA-MB-468 was chosen as it represents a tumor type particularly resistant to treatment (Hudis and Gianni, 2011) and has high endogenous Yap activity (Lamar et al., 2012).

Amot80-CFP and control CFP were stably expressed in MDA-MB-468 cells via lentiviral infection. Whole cell lysates were subjected to immunoblot analysis where relative levels of S127 phosphorylated Yap, total Yap, GAPDH and Amot80-CFP were measured. Amot80 expression resulted in a nearly 50% decrease in the fraction of Yap phosphorylated at serine 127 (Figure 4-1A). S127 phosphorylation of Yap is a key post translational modification which facilitates cytoplasmic sequestration and may promote degradation (Zhao et al., 2010b). Since this dramatic decrease in phosphorylation was observed, the fraction of Yap in the nucleus was measured. MDA-MB-468 cells stably expressing Amot80 were transiently transfected with luciferase constructs including control Renilla luciferase and TEAD-Gal4, Gal4-Firefly



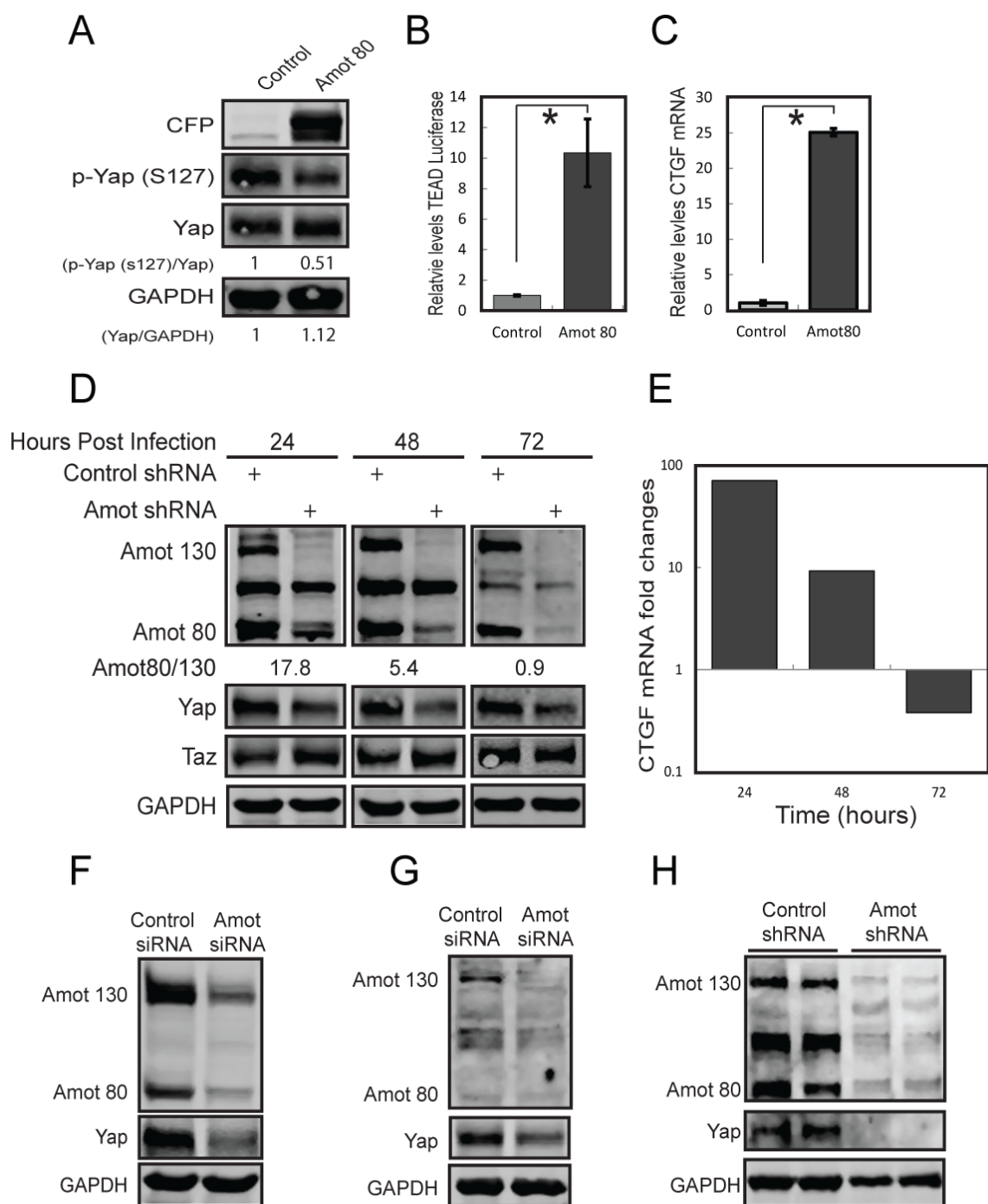
luciferase. Amot80 expressing cells showed a 10-fold increase in TEAD luciferase activity compared to control (Figure 4-1B). Isolated mRNA from these cells was analyzed with qRT-PCR to determine the relative levels of connective tissue growth factor (CTGF), the best described target gene of Yap-TEAD (Zhao et al., 2008b). Cells expressing Amot80 showed a 25-fold increase in CTGF mRNA compared to control (Figure 4-1C).

The requirement of Amot expression for Yap activity was then investigated. A previous report, arguing that Amot is a novel Yap inhibitor, indicated that silencing of Amot expression in HEK 293T cells resulted in increased nuclear Yap activity (Zhao et al., 2011a). While HEK 293T cells have high endogenous levels of both the 130 and 80KDa isoforms of Amot, this report only demonstrated silencing of Amot130 but not Amot80. To address this apparent discrepancy and to investigate whether Yap requires Amot80 for nuclear activity in mammary epithelial cells, Amot isoforms were differentially silenced in MDA-MB-468 cells. Differential silencing of the Amot isoforms was possible due to a previous observation that cells subjected to si- or shRNA against Amot would lose Amot130 expression approximately 24 hours earlier than Amot80. This phenomenon allows for the disentanglement of the relative requirements for Amot130 and Amot80 expression. The differential protein stability of Amot130 vs. Amot80 may be due to the presence of an interaction motif in the N-termini of Amot130. This motif allows interaction with the ubiquitin ligase neural-precursor-cell-expressed developmentally down-regulated (NEDD4). NEDD4 binding to Amot130 promotes degradation (Wang et al., 2012). MDA-MB-468 cells were incubated with lentivirus packaging scramble control or Amot shRNA. Cells were harvested at 24 hour intervals to exploit the disparity between isoform stability. Whole cell lysates were subjected to immunoblot analysis to measure the endogenous levels of Amot130, Amot80, Yap, Taz, and GAPDH. Within 24 hours of exposure to virus containing Amot shRNA, the Amot130 band was undetectable whereas the Amot80 band was apparent although diminished. Amot80 levels were 17 fold higher than Amot130 at the 24hour time point. At 48 hours post infection a much smaller fraction of Amot80 was detectable, however, the ratio of

Amot80 to Amot130 still favored Amot80 by a 5 fold margin. By 72 hours post infection, both isoforms were totally silenced. Total Yap levels decreased at each time point observed while Taz level changes were inconsistent (Figure 4-1D).

These cells differentially expressing Amot80 and Amot130 were also analyzed for relative CTGF mRNA expression. Cells harvested after 24 hours, which were silenced for Amot130 but not Amot80 expression, showed a nearly 70-fold increase in CTGF mRNA levels compared to control. Cells harvested after 48 hours, which had no Amot130 and low Amot80 expression, displayed an approximately 9-fold increase in CTGF mRNA. However, cells harvested after 72 hours, which were completely silenced for Amot130 and Amot80 expression, showed a statistically significant decrease in CTGF mRNA versus control cells (Figure 4-1E). Together, these data strongly indicated Amot80 functions as an activator of Yap and is required for Yap nuclear activity.

The observation in MDA-MB-468 cells that complete silencing of Amot resulted in a decrease of total Yap protein was reproduced in HEK293T cells and in BT474 cells, which have high and only detectable levels of Amot, respectively. Unlike a previous report that found silencing Amot in HEK293T cells (Wang et al., 2012) resulted in no changes in Yap total protein this study finds that Yap levels consistently decreased subsequent to Amot silencing regardless of cell type. Further, HEK293T and BT474 cells transiently transfected with control or siRNA directed against Amot transcripts produced similar results. Briefly, whole cell lysates from these cells were subjected to immunoblot analysis and probed for Amot, Yap and GAPDH levels. Data shown are representative of 4 independent experiments which all demonstrated a decrease in total Yap protein following Amot silencing (Figure 4-1F,G). To further confirm this observation in MDA-MB-468 cells, an shRNA sequence directed against a second region in Amot was used to stably silence Amot expression. Immunoblot analysis of Amot, Yap, and GAPDH levels showed a decrease in Yap total protein following loss of Amot expression (Figure 4-1H).



**Figure 4-1 Amot80 Promotes and is Required for Yap Transcriptional Activity in Mammary Epithelia.**

A. MDA-MB-468 cells were infected with lentivirus containing control CFP or Amot80-CFP. Whole cell lysates were resolved by SDS-PAGE and probed for indicated proteins. Indicated quantification of S127 phosphorylated Yap fraction over total Yap fraction was determined with densitometric analysis and quantified with ImageJ. B. Activation of Yap dependent transcription factor complexes was determined with a dual-luminometer measuring TEAD luciferase activity on cells from A. C. Relative levels of CTGF mRNA transcripts, in cells from A, were measured with qRT-PCR. D. MDA-MB-468 cells were stably infected with lentivirus containing control shRNA or shRNA directed against Amot transcripts. Cells were harvested and subjected to immunoblot analysis at indicated time points subsequent to an eight hour infection. The ratio of Amot80 expression over GAPDH was determined by densitometric analysis and quantified with ImageJ. E. Cells from D were subjected to RNA extraction and qRT-PCR detection of CTGF mRNA transcripts. F. HEK-293T cells were transiently transfected with non-targeting siRNA or siRNA directed against Amot transcripts. Whole cell lysates were subject to immunoblot analysis. G. BT474 cells were treated as in F. H. MDA-MB-468 cells were transiently transfected with non-targeting shRNA or shRNA directed against Amot transcripts and analyzed as in A. Indicated lanes loaded in duplicate. Error bars indicate standard deviation of the mean. \* Indicate p-value <0.01 calculated with unpaired two sample T-Test.

#### 4.2.2. Amot80 Complexes with and Redistributes Yap in Mammary Epithelia.

The observation that Amot80 expression induces Yap nuclear activity prompted an investigation into how this effect was being mediated. Amot80 lacks the PPXY motifs found in Amot130, AmotL1, and AmotL2 that are required to bind WW domains in Yap (Figure 4-2A) (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011a). While Amot family members are capable of both hetero- and homo-dimerization (Yi et al., 2011), there are no reports that directly address Amot80 interactions with all Amot family members. To determine the relative binding affinity of Amot80 for each Amot family member, HEK293T cells were transiently transfected with Myc and Flag-tagged Amot80, Amot130, AmotL1, and AmotL2. These cells were subjected to Myc immunoprecipitation followed by Flag immunoblot analysis. This revealed that Amot80 binds itself, Amot130, and AmotL1, but not AmotL2. Amot130 was further shown to bind to itself and AmotL2 weakly, but associated strongly with AmotL1. AmotL1 and AmotL2 were also found to homo-dimerize (Figure 4-2B). Since Amot80 formed complexes with Amot130 and AmotL1, but not L2, only the interactions of Amot130 and AmotL1 with Amot80 were investigated.

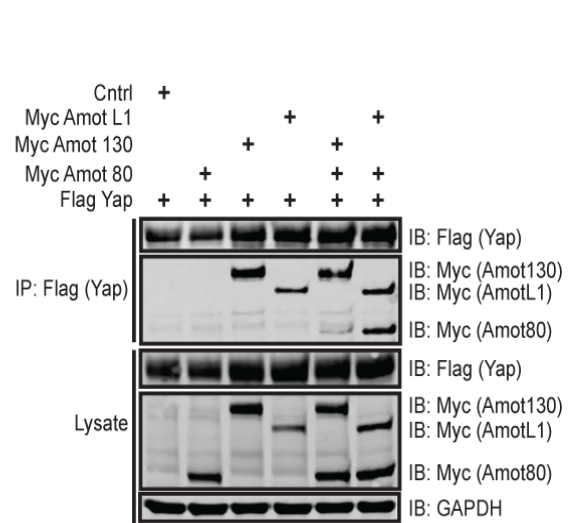
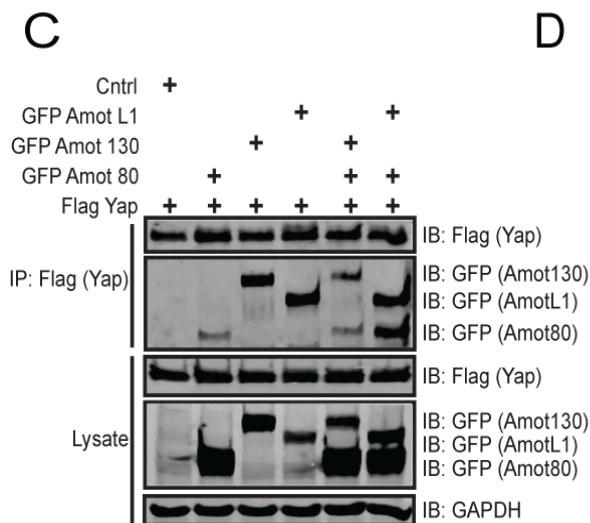
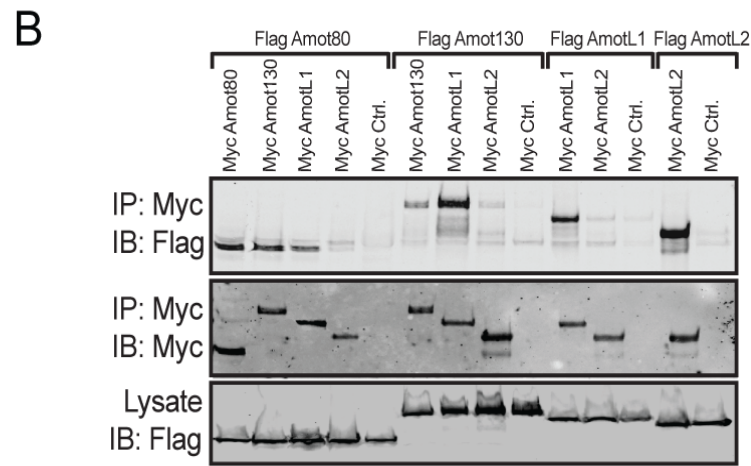
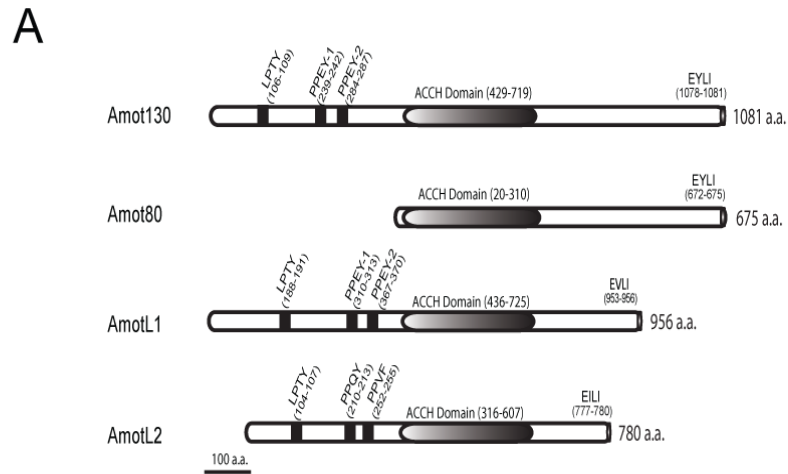
Amot80 expression in MDA-MB-468 cells resulted in significant increases in nuclear Yap activity. Given the ability of Amot80 to heterodimerize with Amot130 and AmotL1, the question of whether Amot80 was able to complex with Yap through Amot130 or AmotL1 was investigated. MDA-MB-468 cells were infected with lentivirus carrying green fluorescent protein (GFP) tagged Amot family members and Flag tagged Yap or control Flag or GFP. These cells were immunoprecipitated with anti-Flag antibody and immunostained with antibodies against Flag and GFP. Amot80 marginally bound Yap, whereas Amot130 and AmotL1 strongly associated. However, co-expression of Amot80 with Amot130 resulted in similar Yap association of each whereas co-expression of Amot80 with AmotL1 markedly increased Amot80 association with Yap (Figure 4-2C).

The small but detectable association of Amot80 with Yap without co-expression of AmotL1 or Amot130 may be explained by the presence of endogenous Amot130 and AmotL1 in MDA-MB-468 cells. To explore this possibility, MCF7 cells which lack detectable expression of Amot130, were transiently transfected with Myc tagged Amot family members and Flag tagged Yap. In MCF7 cells, expressed Amot130 and AmotL1 complexed with Yap. Amot80, however, was observed to complex with Yap only when co-expressed with Amot130 and AmotL1 (Figure 4-2D). These results suggest that Amot80 cannot directly bind Yap but rather indirectly through AmotL1 and to a lesser extent Amot130.

Given the ability of Amot80 to induce redistribution of apical polarity proteins from junctions to intracellular recycling compartments (Heller et al., 2010; Wells et al., 2006), the effect of Amot80 expression on Yap localization was investigated. CFP tagged Amot80 was stably expressed in MDA-MB-468 cells alone and in combination with YFP tagged Amot130 and AmotL1. These cells were fixed and immunostained for endogenous Yap then subjected to immunofluorescence analysis. Yap expression in MDA-MB-468 cells was relatively diffuse (Figure 4-2E 1<sup>st</sup> column). Amot80 expression resulted in the sequestration of Yap into intracellular compartments that contain Amot80 reminiscent of those previously observed (Figure 4-2E 2<sup>nd</sup> column) (Heller et al., 2010). Conversely, expression of Amot130 resulted in recruitment of Yap to cell-cell contacts as previously demonstrated in MDCK cells (Figure 4-2E 3<sup>rd</sup> column) (Zhao et al., 2011a). While AmotL1 expression was diffuse, it appeared to codistribute with Yap (Figure 4-2E 4<sup>th</sup> column). Coexpression of Amot80 with Amot130 as well as Amot80 with AmotL1 dominantly retargeted Amot130/L1 and endogenous Yap into intracellular compartments (Figure 4-2E 5<sup>th</sup> and 6<sup>th</sup> columns).

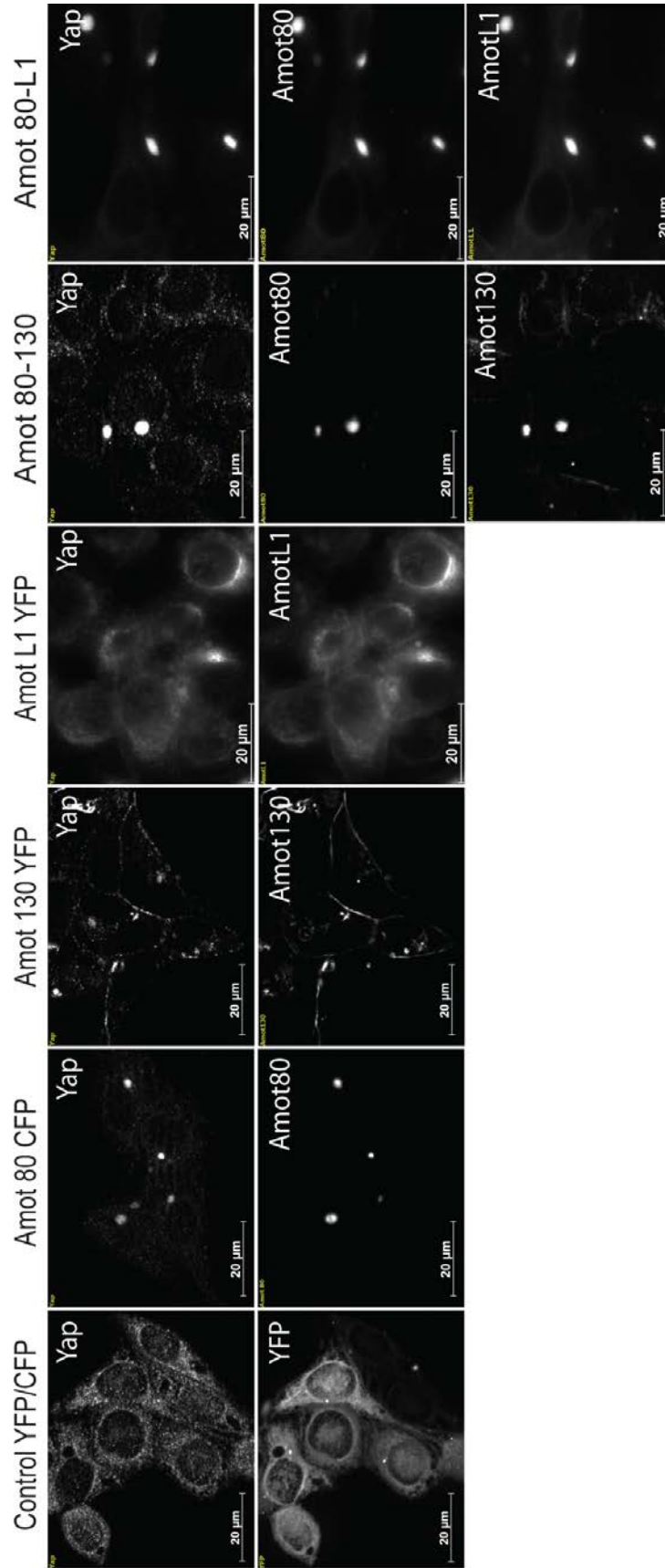
Taken together these data suggest that Amot80 is able to complex with Yap in an Amot130 and AmotL1 dependent manner. In MDA-MB-468 cells, that have detectable levels of endogenous Amot80, Amot130, and AmotL1, control cells showed a diffuse pattern of Yap expression. Exogenous expression of Amot80, however, resulted in the dominant redistribution

of Yap regardless of increased levels of Amot130 and AmotL1. These findings suggest that Amot80 may be a dominant factor in Yap localization and activity.





E



**Figure 4-2 Amot80 Dominantly Reorganizes Yap Complexes in an Amot130/L1 Dependent Manner.**

A. Illustration of the domain architecture of members of the Amot family (Figure made by Jacob Adler). B. HEK-293T cells were transiently transfected with indicated plasmids. MYC tagged proteins were immunoprecipitated. Precipitates and whole cell lysates were subjected to immunoblot analysis. C. MDA-MB-468 cells were stably infected with lentivirus containing indicated plasmid vectors. Immunoprecipitation of Flag tagged Yap was performed. Precipitates and whole cell lysate were subjected to immunoblot analysis. D. MCF7 cells were transiently transfected with indicated plasmid vectors and analyzed as in C. E. MDA-MB-468 cells were stably infected with lentivirus containing CFP tagged Amot80 alone or in combination with YFP tagged Amot130 or YFP tagged AmotL1. Cells were fixed and stained for endogenous Yap then subjected to immunofluorescence analysis.

#### 4.2.3. Amot80 Requires a PDZ Binding Motif to Reduce Phosphorylation of Yap on S127.

A requirement for the PDZ binding domain in Amot80 has been demonstrated in several contexts including direction of chemotactic cell migration (Levchenko et al., 2003), disruption of apical polarity (Wells et al., 2006), and promotion of prolonged Erk1/2 phosphorylation (Ranahan et al., 2011). However, genetic expression of Amot80 $\Delta$ C results in embryonic lethality in mice (Levchenko et al., 2003). In an effort to further clarify the contexts in which Amot80 requires its polarity protein interaction motif, the effects of Amot80 $\Delta$ C expression on Yap activity and localization were investigated. MDA-MB-468 cells were stably infected with lentivirus containing Amot80-CFP, Amot80 $\Delta$ C-CFP, and control CFP. Whole cell lysates from these cells were subjected to immunoblot analysis and probed for CFP, Yap phosphorylated on S127, total Yap, and GAPDH. The expression of Amot80 reduced the fraction of Yap phosphorylated on S127, while expression of Amot80 $\Delta$ C failed to do so (Figure 4-3A). These cells were also subjected to qRTPCR analysis to determine relative levels of mRNA produced from Yap/TEAD transcriptional activity. In cells stably expressing Amot80, a nearly 8-fold increase in CTGF levels was detected while cells expressing Amot80 $\Delta$ C displayed a nearly 4-fold increase compared to control cells (Figure 4-3B). Relative levels of the Yap-TEAD target cysteine-rich angiogenic inducer 61 (Cyr61), were also quantified with qRTPCR. Cells stably expressing Amot80 had a 15-fold increase in Cyr61 mRNA levels while Amot80 $\Delta$ C expressing cells had a 5-fold increase compared to control cells (Figure 4-3C).

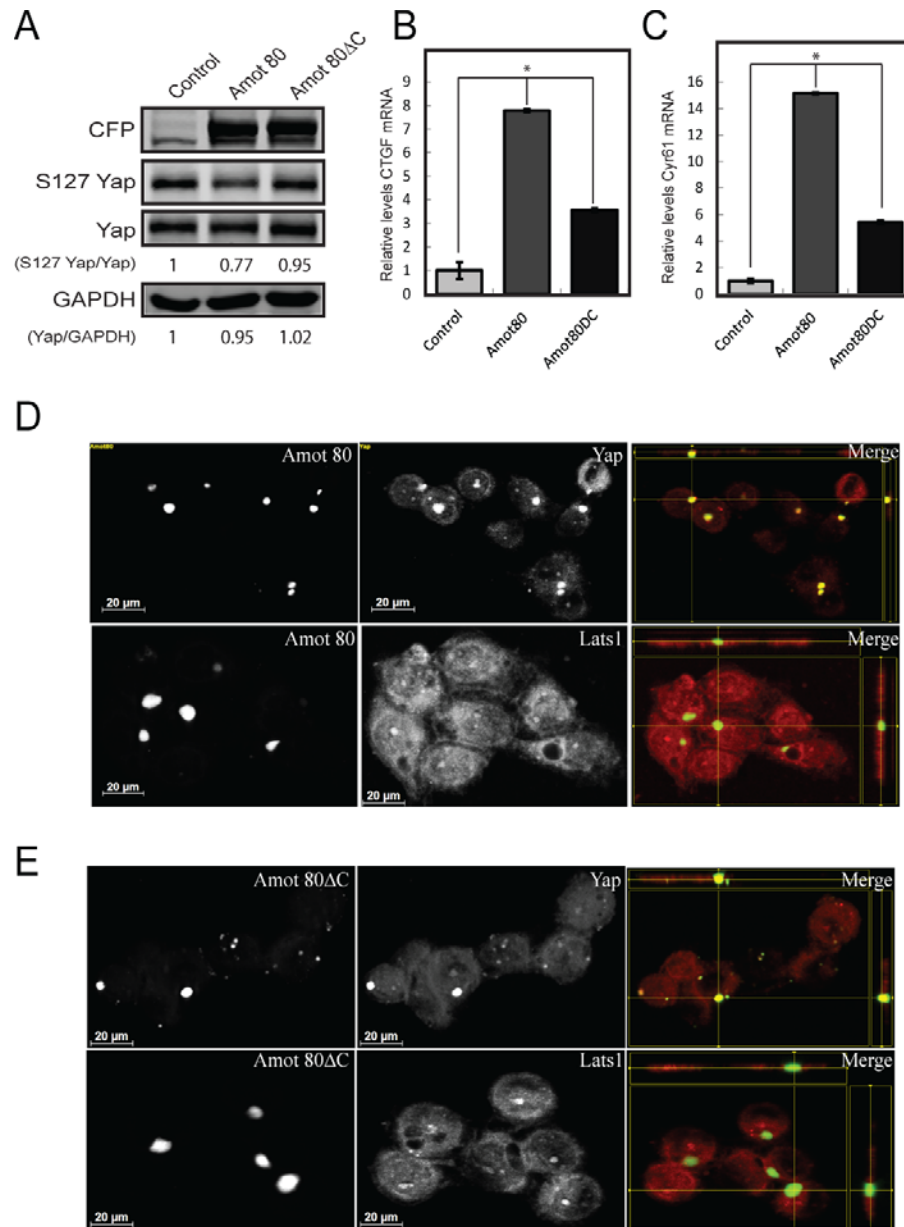
Thus the interaction of Amot80 with polarity proteins partly underlies its ability to activate Yap. Inhibition of Yap nuclear activity has been demonstrated to involve the Hippo effector kinases Lats1/2 and their phosphorylation of Yap on S127 (Hao et al., 2008; Xu et al., 1995). Amot80 expression reduces the fraction of Yap phosphorylated on S127. Whether this reduction was effected by direct sequestration of Yap from Lats or indirect inhibition of Lats

kinase activity was next investigated. MDA-MB-468 cells were stably infected with lentivirus packaging Amot80-CFP, Amot80 $\Delta$ C-CFP, or control CFP. These cells were fixed and immunostained for endogenous Yap and Lats1 proteins. Amot80 expressing cells dominantly redistributed Yap to intracellular compartments where Lats1 was undetectable (Figure 4-3D). Cells stably expressing Amot80 $\Delta$ C also dominantly reorganized Yap into internal puncta where Lats1 was undetectable (Figure 4-3E).

The finding that Amot80 $\Delta$ C induces nuclear Yap activity to a lesser extent than WT Amot80 suggests that Amot80 may have dual roles in regulation of Yap (Figure 4-4). In epithelial cells Amot80 expression disrupts apical polarity in a manner requiring a PDZ binding domain (Wells et al., 2006). Hippo signaling is thought to require apical junctional stability in order to facilitate Yap phosphorylation (Boggiano and Fehon, 2012; Ling et al., 2010). Cells at low confluence have low Hippo signaling and nuclear localized Yap. As cell-cell contacts mature, Hippo signaling increases and Yap is either sequestered or degraded (Zhao et al., 2011b). Cancer cells survive, in part, by disrupting apical polarity which helps maintain sensitivity to growth signaling pathways (Martin-Belmonte and Perez-Moreno, 2012). Loss of apical junctional integrity may therefore facilitate Yap nuclear activity by disrupting Hippo signaling.

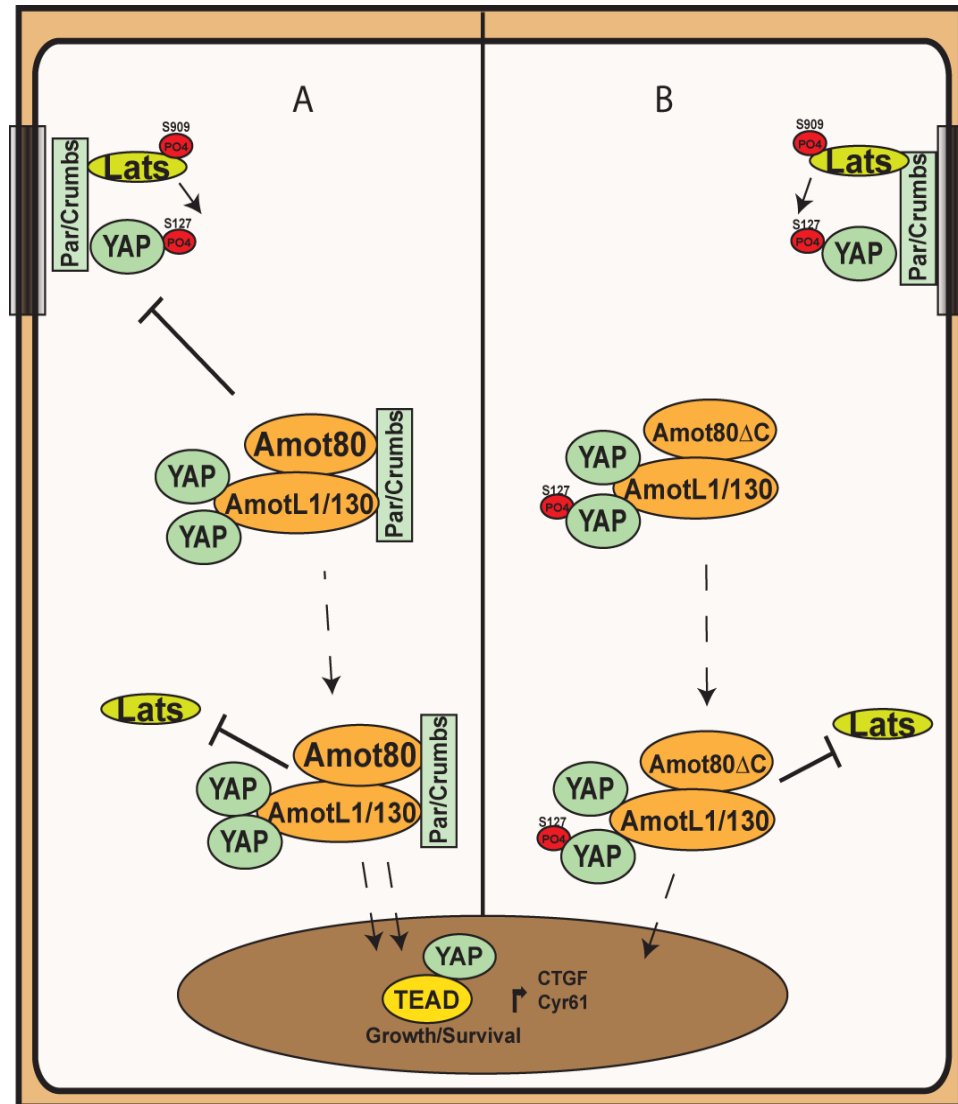
Amot80 disrupts apical polarity and may therefore be responsible for global dampening of Hippo signaling. Amot80 $\Delta$ C, which is unable to disrupt apical polarity, is still able to co-distribute with Yap in Lats1 excluded structures. Amot80 $\Delta$ C, however, does not reduce global levels of Yap phosphorylated on S127. These data suggest the physical redistribution of Yap by Amot80 is only partially responsible for the observed increase in nuclear activity. It is also noteworthy that Amot80 expression in mammary cells harvested at low density show no significant increase in Yap activity compared to control. This observation is consistent with Amot80 functioning as an inhibitor of polarity. Cells at low density do not have matured cell-cell contacts which are necessary for intracellular polarization.

Mammary cell lines differ widely with regard to their apical-basal organization and may therefore regulate Yap activity uniquely. For example, MDA-MB-468 cells have relatively high levels of the tight junction transmembrane protein occludin (Ranahan et al., 2011). Occludin distribution in MDA-MB-468 cells is mainly cytosolic rather than junctional, thus these cells are poorly polarized. In an effort to determine whether Amot80 induced Yap nuclear activity is cell-type specific two additional cell lines were investigated.



**Figure 4-3 Amot80 Requires PDZ Binding Domain to Reduce Yap S127 Fraction, but not to Exclude Lats1 from Puncta.**

A. MDAMB-468 cells were infected with lentivirus containing control, Amot80, or Amot80 lacking the four extreme C-terminal amino acid residues (Amot80 $\Delta$ C). Whole cell lysates were resolved by SDS-PAGE and probed for indicated proteins. Indicated quantification of S127 Yap fraction over total Yap fraction was determined with densitometric analysis and quantified with ImageJ. B-C. mRNA was extracted from cells from A and subjected to qRT-PCR analysis. Relative levels of CTGF and Cyr61 mRNA transcripts were measured as indicated. D. Cells from A stably expressing Amot80-CFP were fixed and immunostained for expression of endogenous Yap and Lats1. E. Cells from A stably expressing Amot80 $\Delta$ C-CFP were fixed and immunostained for expression of endogenous Yap and Lats1. Error bars indicate standard deviation of the mean. \* Indicate p-value <0.01 calculated with unpaired Student's T-Test.



**Figure 4-4 Model for Dual Roles of Amot80 in Yap Regulation.**

A. Amot80 heterodimerizes with Amot130 and AmotL1. All Amot family members contain PDZ binding domains which enable association of Amots with polarity proteins in the Crb and Par complexes. Amot130 and AmotL1 contain N-terminal PPEY motifs which enable binding to WW domains in Yap proteins. Amot80 expression results in internalization of both polarity proteins and Yap. Redistribution of Crb and Par components results in disruption of apical junction integrity. Disruption of apical junctional integrity compromises global Hippo signaling resulting in decreased Yap phosphorylation on serine 127. Amot80 induced internalized Yap is sequestered from Lats and available for nuclear import. B. Amot80 $\Delta$ C lacks the motif required for association with apical polarity proteins. Amot80 $\Delta$ C still heterodimerizes with Amot130 and AmotL1 and still internalizes Yap. Failure to disrupt apical junctions results in normal Hippo signaling thus a greater fraction of Yap is phosphorylated on serine 127. Yap associated with Amot80 $\Delta$ C is still sequestered from Lats and therefore remains unphosphorylated on serine 127 and available for nuclear import.

#### 4.2.4. Amot80 Enhances Yap Transcriptional Activity Across Mammary Cell Types.

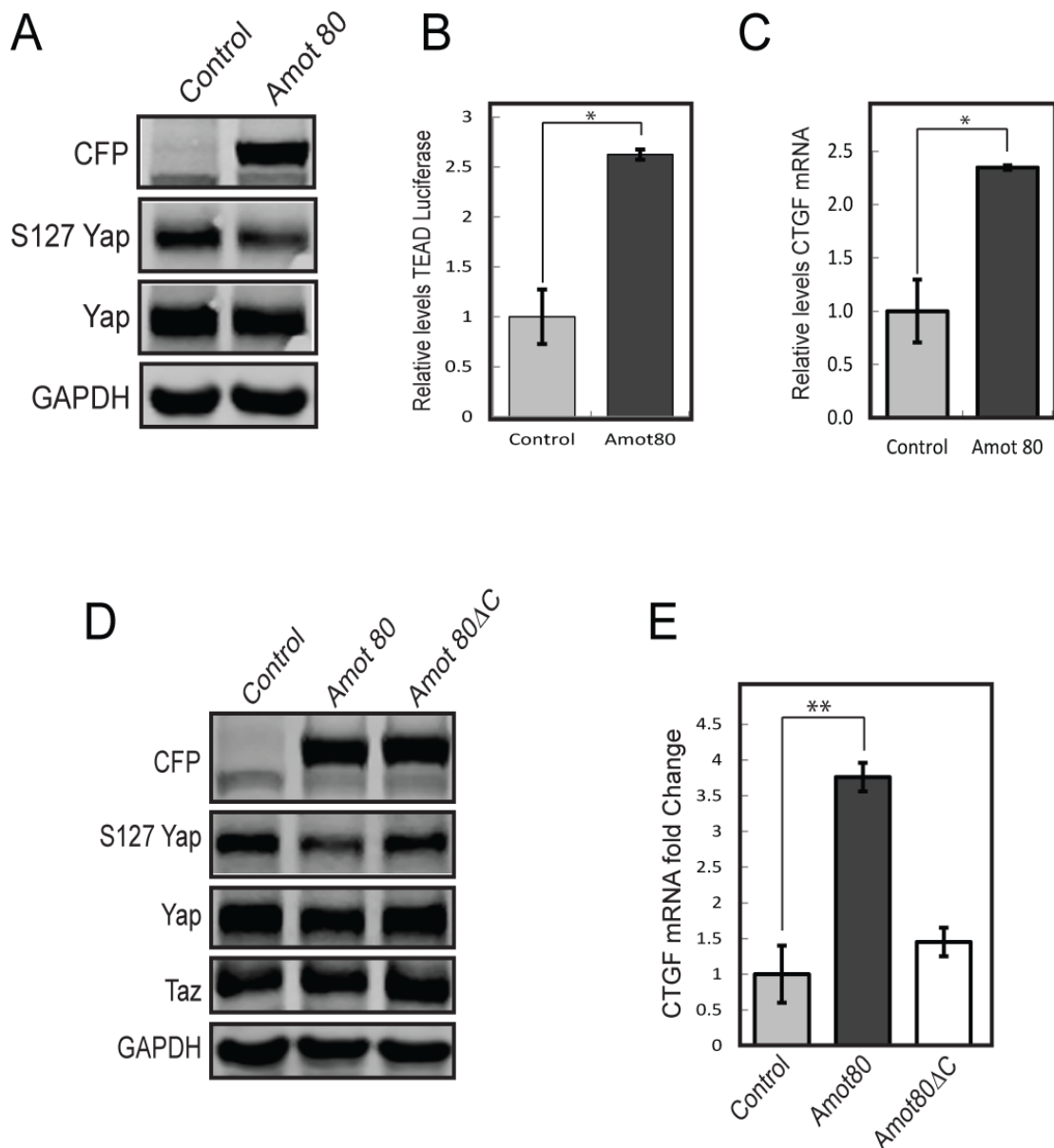
MDA-MB-468 cells represent a basal, adenocarcinoma, ER-PR-Her2- tumor type with poor patient outcome (Shastry and Yardley, 2013). These cells express both isoforms of Amot (Ranahan et al., 2011), and are highly responsive to changes in the ratio of Amot80 to Amot130 /AmotL1 with respect to Yap nuclear activity (Figure 4-1). To determine if Amot80 induced Yap nuclear activity is a cell type specific phenomenon, two additional cell types were selected based on growth receptor status, invasive capacity, and Amot expression. BT474 cells represent a luminal tumor type derived from an invasive ductal carcinoma. These cells are ER+PR+Her2+ and express low levels of both isoforms of Amot (Kao et al., 2009; Ranahan et al., 2011). Lastly the cell line MCF10A was examined. MCF10A cells were derived from the breast of a donor with fibrocystic disease. These cells spontaneously immortalized, are non-transformed, and do not form tumors in mice (Debnath et al., 2003). Amot expression is undetectable in MCF10A via immunoblot analysis with RIPA extraction but is detectable following 8M urea fractionation.

BT474 cells were stably infected with lentivirus carrying Amot80-CFP or control CFP. These cells were harvested for immunoblot analysis and probed for expression of CFP, S127 phosphorylated Yap, total Yap, and GAPDH. As was observed in MDA-MB-468 cells, BT474 cells stably expressing Amot80 reduced the fraction of Yap phosphorylated at S127 (Figure 4-5A). These cells were next transiently transfected with luciferase constructs including control Renilla luciferase and TEAD-Gal4, Gal4-Firefly luciferase. Amot80 expressing cells showed a 2.5-fold increase in TEAD luciferase activity compared to control (Figure 4-5B). mRNA from these cells was analyzed with qRT-PCR to determine relative levels of connective tissue growth factor CTGF. Cells expressing Amot80 showed a nearly 2.5-fold increase in CTGF mRNA compared to control (Figure 4-5C). These data suggest that Amot80 expression induces increased Yap transcriptional activity independent of the growth requirements of the mammary tumor type.

The ability of Amot80 expression to induce Yap transcriptional activity was next tested in a non-tumorigenic mammary cell type. MCF10A cells were stably infected via lentivirus with



Amot80-CFP, Amot80 $\Delta$ C-CFP, and control CFP. Whole cell lysates were resolved by SDS-PAGE and subjected to immunoblot analysis. Relative levels of CFP, total and S127 phosphorylated Yap, Taz, and GAPDH were probed. Consistent with data observed in MDA-MB-468 cells, MCF10A cells expressing Amot80, but not Amot80 $\Delta$ C or control CFP decreased the fraction of S127 phosphorylated Yap (Figure 4-5D). These cells were analyzed for CTGF mRNA levels via qRT-PCR. Cells expressing Amot80 showed a nearly 4-fold increase in CTGF mRNA while cells expressing Amot80 $\Delta$ C had no significant difference in CTGF mRNA compared to control (Figure 4-5E). The disparity between Amot80 $\Delta$ C expression in MDA-MB-468 and MCF10A cells with respect to Yap nuclear activity as measured by CTGF mRNA levels, again suggests dual roles for Amot80. In order to disentangle the relative roles of Amot80 in mediating Yap nuclear activity, a model system with greater cell polarity and growth inhibition, and presumably greater sensitivity to Hippo signaling, was used and forms the basis of the following section of this dissertation. MCF10A cells are non-transformed and when seeded into Matrigel<sup>TM</sup> form polarized, growth arrested acini (Petersen et al., 1992). Therefore, this model was exploited to clarify the relative contributions of modified Hippo signaling vs. physical redistribution of Yap by Amot80.



**Figure 4-5 Amot80 Reduces the Fraction of Yap Phosphorylated at S127 and Increases Yap Transcriptional Activity Across Mammary Cell Types.**

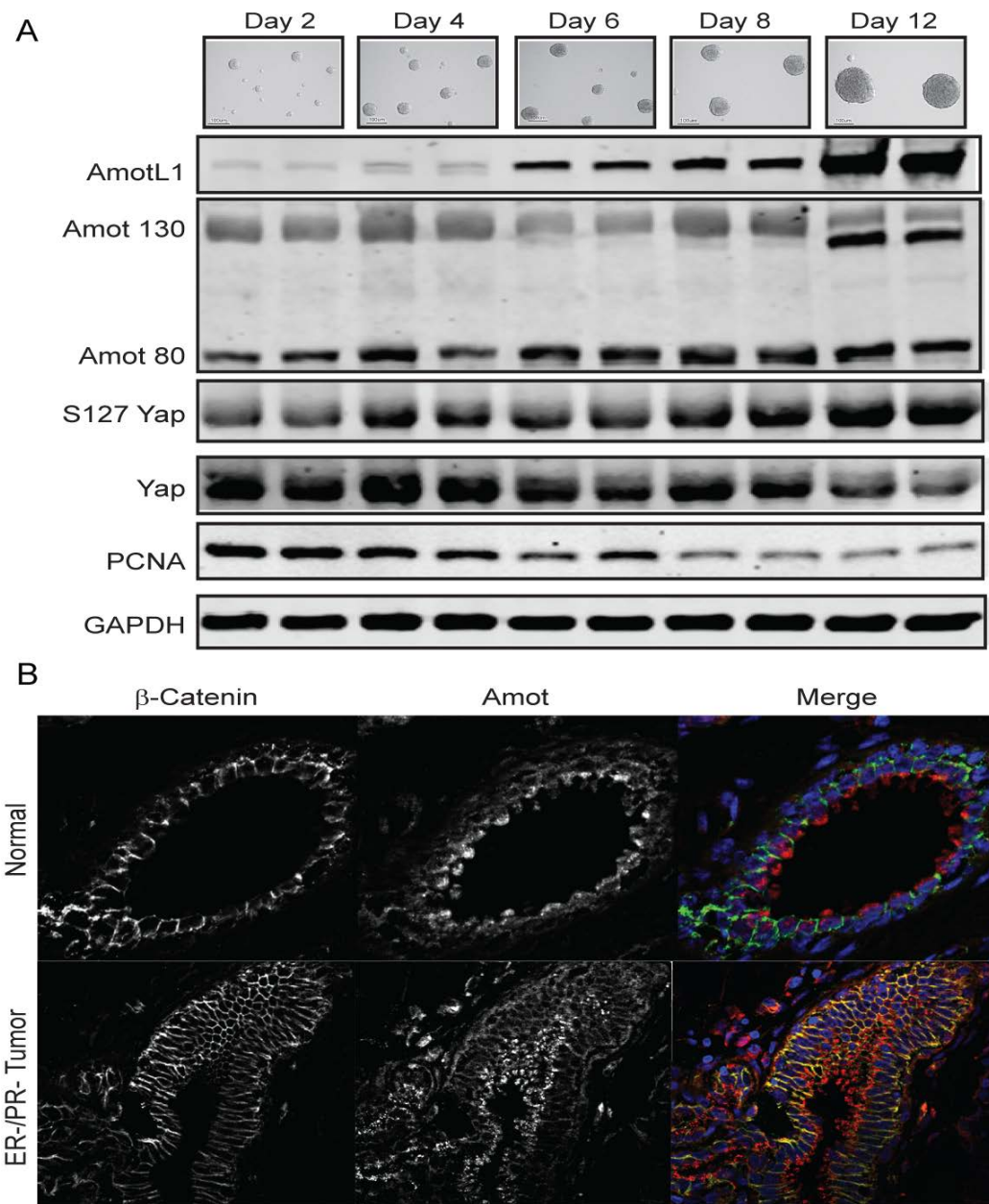
A. BT474 cells were infected with lentivirus containing control CFP or Amot80-CFP. Whole cell lysates were resolved by SDS-PAGE and probed for indicated proteins. B. Activation of Yap dependent transcription factor complexes was determined with a dual-luminometer measuring TEAD luciferase activity on cells from A. C. Relative levels of CTGF mRNA transcripts, in cells from A, were measured with qRT-PCR. D. MCF10A cells were infected with lentivirus containing control CFP, Amot80-CFP or Amot80 lacking the four extreme C-terminal amino acid residues (Amot80 $\Delta$ C). Whole cell lysates were resolved by SDS-PAGE and probed for indicated proteins. E. Relative levels of CTGF mRNA transcripts, in cells from D, were measured with qRT-PCR. Error bars indicate standard deviation of the mean. \* Indicate p-value <0.01 \*\* Indicate p-value <0.05 calculated with unpaired Student's T-Test.

#### 4.2.5. Amot Expression During Mammary Acini Formation and in Human Tissue

##### Samples.

Consistent with Amot80 promoting prolonged Erk1/2 phosphorylation in MCF7 cells, Amot80 expression in MCF10A cells seeded into Matrigel<sup>TM</sup> results in acini overgrowth with apparent luminal filling (Ranahan et al., 2011). Amot expression is not detectable in MCF10A cells grown on plastic substrata, however, the genetic expression profile of MCF10A cells changes dramatically when grown in Matrigel<sup>TM</sup> (Debnath et al., 2003). In order to determine what role, if any, Amot family members play in mammary acini formation MCF10A cells were seeded into Matrigel<sup>TM</sup> and harvested over a time course. MCF10A cells were observed over a 12 day period. Stereomicroscopic images were taken at indicated time points. Acini from indicated time points were harvested for immunoblot analysis and relative levels of Amot, AmotL1, total and S127 phosphorylated Yap, proliferating cell nuclear antigen (PCNA), and GAPDH were determined. Acini were observed to grow as a ball of cells until around day 7 when growth arrest was apparent (Figure 4-6A top row). AmotL1 expression was barely detectable until day 6 when it began to dramatically increase up until day 12. Amot80-130 expression was apparent consistently across all time points with a lower Amot 130 band becoming apparent on day 12. Total Yap protein steadily decreased from day 2-12 whereas Yap phosphorylated at S127 increased over that time. PCNA expression correlated with an early growth phase followed by a marked decrease during the differentiation phase (day 8-12) (Figure 4-6A bottom rows).

In collaboration with Lauren Bringman, endogenous Amot expression was also measured in human mammary tissues. Both normal and tumor tissue was fixed and stained for adherens junctional marker  $\beta$ -Catenin and Amot. Amot was observed to have an apical distribution in the monolayer of epithelial cells facing the ductal lumen in normal tissues (Figure 4-6B top row). Amot expression in tumor tissues was much less organized. Amot in tumor tissues localized sporadically within the luminal epithelial cells and was strikingly apparent in the basal layer and surrounding stroma (Figure 4-6B bottom row).



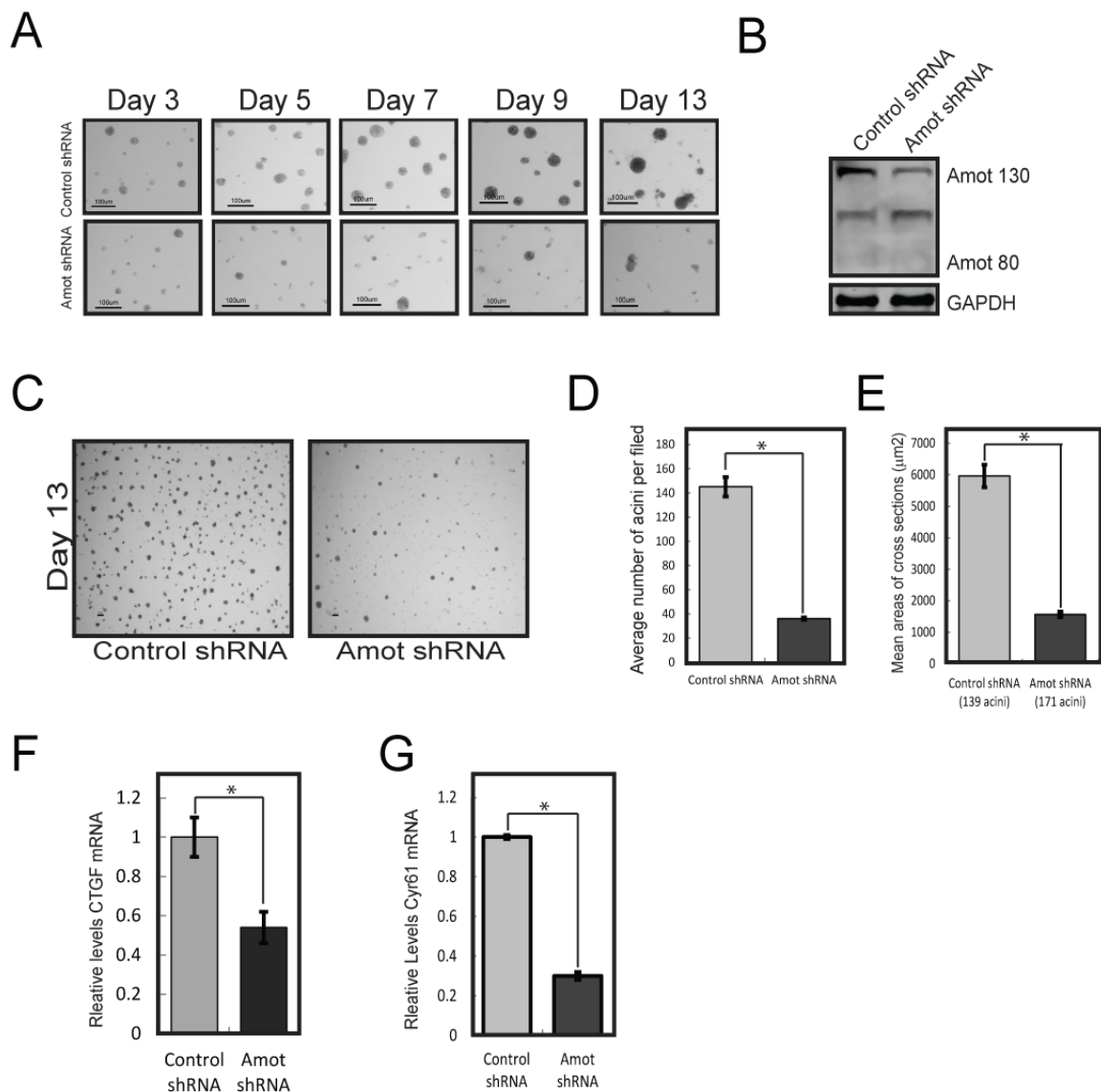
**Figure 4-6 Endogenous Amot Expression During Acini Formation and in Human Mammary Tissues.**

A. (Top row) MCF10A cells were processed as previously described (Ranahan et al., 2011). Briefly, cells were seeded onto matrigel (BD Bioscience) coated 35mm dishes. Stereoscopic images were taken at indicated time points. (Bottom rows) Acini were harvested out of matrigel and subjected to immunoblot analysis as described in (Ranahan et al., 2011). Briefly, acini cells were sheered with 27G needle, proteins were extracted with PLC lysis buffer. Matrigel/ lysed cell slurry was allowed to depolymerize on ice for 20 minutes before high centrifugation. Protein levels were normalized via LDH assay per manufacturer's instructions. B. Normal and tumor mammary tissue sections were subjected to de-paraffinization and slide processing. Sections were then immunolabelled with Amot and  $\beta$ -catenin. Nuclei were stained with Hoechst. Section (B) completed by with Lauren Bringman.

#### 4.2.6. Amot is Required for Yap Transcriptional Activity and Cell Growth During Acini Development.

The discovery that endogenous Amot is upregulated during acini formation confirmed the previous observation that Amot expression is required for Erk1/2 activity in mammary epithelia (Ranahan et al., 2011). Acini formation requires Erk1/2 activity, and failure to temporally regulate Erk1/2 results in luminal filling and acini outgrowth (Turley et al., 2008). Amot expression was visible within 2 days of seeding into Matrigel™ and remained constant throughout the experimental timeframe. Since Amot expression is required to maintain Yap transcriptional activity in cells grown on plastic substrata, a requirement for Amot expression in Matrigel™ was examined.

MCF10A cells were seeded into Matrigel™ and given 18 hours to adhere before being stably infected with lentivirus containing scramble control shRNA or shRNA directed against Amot transcripts. These cells were imaged with a stereomicroscope over a time course. MCF10A cells silenced for Amot expression displayed gross growth defects as early as day 3 compared to control acini (Figure 4-7A). MCF10A cells on plastic were also infected with control and Amot shRNA and subjected to immunoblot analysis to demonstrate loss of Amot expression (Figure 4-7B). Four wide view sections of control and Amot silenced acini from day 13 (Figure 4-7C) were quantified. Amot silenced acini were fewer in number and smaller in size than control infected acini. Both the average number of acini per field (Figure 4-7D) and average size of acini per field (Figure 4-7E) were calculated. Control and Amot silenced acini were harvested on day 13 and subjected to RNA extraction and generation of cDNA for qRT-PCR analysis. Levels of CTGF (Figure 4-7F) and Cyr61 (Figure 4-7G) transcripts were determined. In acini stably silenced for Amot expression both CTGF and Cyr61 mRNA levels were significantly lower. These data confirm a role for Amot in mediating Yap nuclear activity both in tumorigenic cell lines grown on plastic substrata and non-tumorigenic acini grown in Matrigel™. Overall these results indicate Amot is required for mediation of Yap transcriptional activity.

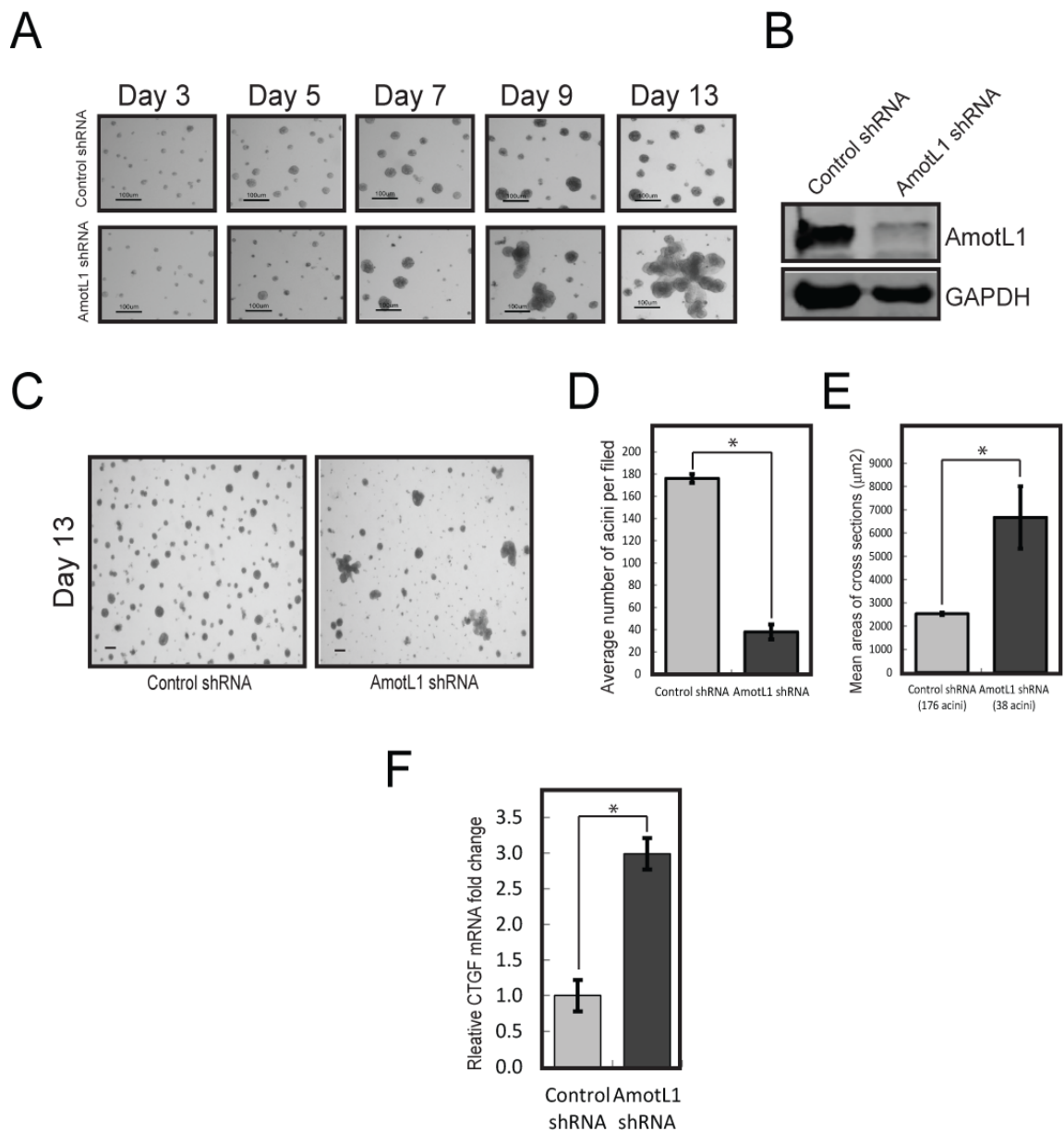


**Figure 4-7 Amot is Required for Yap Transcriptional Activity and Cell Growth During Acini Development.**

A. MCF10A cells seeded into matrigel were infected with lentivirus containing control shRNA or shRNA directed against Amot transcripts. Stereoscopic images were taken at indicated time points. B. Cells from A were subjected to immunoblot analysis and probed for indicated proteins. C. Wide field stereoscope images were taken which represent one of the four fields used to generate the data graphed in D. D. Four fields as indicated in C were analyzed for average acini number E. Four fields as indicated in C were analyzed for average size ( $\mu$ m<sup>2</sup>). F. Cells from A were analyzed for relative mRNA transcripts of CTGF. G. Cells from A were analyzed for relative mRNA transcripts of Cyr61. Error bars indicate standard deviation of the mean. \* Indicate p-value < 0.01 calculated with unpaired Student's T-Test.

#### 4.2.7. Loss of AmotL1 results in increased Yap transcriptional activity and acini overgrowth.

MCF10A cells grown on plastic substrata have high levels of AmotL1 expression. Levels of AmotL1 expression were initially down-regulated when MCF10A cells were seeded into Matrigel<sup>TM</sup> unlike Amot80/130 which were upregulated and remained constant. These data suggested that Amot may be playing a role in driving basal growth, whereas AmotL1 is likely involved with differentiation. Acini begin to differentiate around day 6 and continue to do so until quiescence is achieved around day 12 (Debnath and Brugge, 2005; Debnath et al., 2003). AmotL1 expression was upregulated around day six and dramatically increased until day 12 suggesting a causative role in differentiation. To address this potential role, AmotL1 expression was silenced during acini formation. MCF10A cells were seeded into Matrigel<sup>TM</sup> and given 18 hours to adhere before being stably infected with lentivirus expressing scramble control shRNA or shRNA directed against AmotL1 transcripts. These cells were then imaged with a stereomicroscope over a time course. MCF10A cells silenced for AmotL1 displayed normal growth until around day 6 when overgrowth became apparent and continued until termination of the experiment on day 13 (Figure 4-8A). MCF10A cells in Matrigel<sup>TM</sup> were infected with control and shRNA directed against AmotL1 and subjected to immunoblot analysis to measure loss of AmotL1 protein (Figure 4-8B). Four wide view sections of control and AmotL1 silenced acini from day 13 (Figure 4-8C) were quantified. AmotL1 silenced acini were fewer in number but much larger in size than control infected acini. Both the average number of acini per field (Figure 4-8D) and average size of acini per field (Figure 4-8E) were calculated. Control and AmotL1 silenced acini were harvested on day 13 and subjected to RNA extraction and generation of cDNA for qRT-PCR analysis to measure the levels of CTGF mRNA (Figure 4-8F). In acini stably silenced for AmotL1 expression CTGF was significantly higher. These results suggest a role for AmotL1 in suppression of Yap transcriptional activity and promotion of growth control during acini development.



**Figure 4-8 Loss of Amotl1 Results in Increased Yap Transcriptional Activity and Acini Overgrowth.**

A. MCF10A cells seeded into matrigel were infected with lentivirus containing control shRNA or shRNA directed against AmotL1 transcripts. Stereoscopic images were taken at indicated time points. B. Cells from A were subjected to immunoblot analysis and probed for indicated proteins. C. Wide field stereoscope images were taken which represent one of the four fields used to generate the data graphed in D. D. Four fields as indicated in C were analyzed for average acini number E. Four fields as indicated in C were analyzed for average size ( $\mu\text{m}^2$ ). F. Cells from A were analyzed for relative mRNA transcripts of CTGF. Error bars indicate standard deviation of the mean. \* Indicate p-value <0.01 calculated with unpaired Student's T-Test.



### 4.3. DISCUSSION

#### 4.3.1. Differential Roles for Amot Family Members in the Regulation of Yap Transcriptional Activity.

This study finds distinct patterns of localization, signaling, and cellular function for Amot80, Amot130, and AmotL1. Amot130 recruits Yap to cell-cell contacts, whereas loss of AmotL1 leads to acini overgrowth. These data are consistent with Amot130 and AmotL1 functioning as negative regulators of Yap as has been previously suggested (Chan et al., 2011; Paramasivam et al., 2011; Wang et al., 2011; Zhao et al., 2011a). Coexpression of Amot80 with Amot130 or AmotL1 results in dominant re-localization of both Amot130/AmotL1 and associated Yap to internal juxtanuclear recycling endosomes. MDA-MB-468 cells have relatively high levels of endogenous Amot and AmotL1 therefore expression of Amot80 alone results in dominant redistribution of Yap. While expression of Amot130 and AmotL1 reduce Yap transcriptional activity (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011a), Amot80 enhances Yap directed transcription of target genes. These findings are consistent with a previous report indicating that the ratio of Amot80 to Amot130 determines whether endothelial cells will be migratory or stable. Endothelial cells are migratory while the relative amount of Amot80 protein is greater than that of Amot130 (Ernkvist et al., 2008). Taken together these observations suggest Amot80 functions as a negative regulator of Amot130 and AmotL1 with respect to control of Yap transcriptional activity.

The ability to differentially silence Amot isoforms enables their respective roles to be elucidated. Loss of Amot130 results in dramatic increases in nuclear Yap activity but only when Amot80 is present. Loss of both Amot isoforms results in loss of Yap transcriptional activity. These data suggest an Amot80 requirement for Yap nuclear activity and sensitivity to the stoichiometry of Amot130 and Amot80.

Although Amot80 did not predominantly immunoprecipitate with AmotL1 in 293T cells, both of these proteins are prevalent in Yap complexes regardless of cell type or expression method. All cell types where Amot80 was expressed have detectable AmotL1 expression therefore it is difficult to distinguish the relative contributions of AmotL1 and Amot80 to Yap regulation. For instance, detection of AmotL1 expression increases dramatically in mammary acini undergoing differentiation, whereas loss of AmotL1 results in normal growth until acini begin to growth arrest. Amot80 is expressed throughout the acini formation process and is presumably driving proliferation since overexpression of Amot80 results in unregulated growth (Ranahan et al., 2011). Since the phenotypes of AmotL1 loss and Amot80 expression are similar, the ratio of AmotL1 to Amot80 may be a deciding factor in whether acini grow or differentiate. In order to determine if Amot80 expression can dominantly overcome AmotL1 mediated acini differentiation, Amot80 would need to be expressed in fully differentiated acini, rather than constitutive Amot80 expression from day 1. Amot80 driven by an inducible promoter would allow expression to be increased following acini maturation.

#### 4.3.2. Mechanism of Induced Yap Transcriptional Activity by Amot80.

Amot80 indirectly associates with Yap presumably through direct interactions with Amot130 and AmotL1. Amot80 also directly associates with multiple-PDZ domain containing proteins Mupp1 and PatJ through its C-terminal PDZ binding domain (Wells et al., 2006). These interactions may facilitate peripheral Yap interactions as well since both Patj and Mupp1 directly bind Yap (Varelas et al., 2010). This possibility could be tested by silencing PatJ or Mupp1 expression in cells where Amot80 is driving Yap activity. Since Amot130 and AmotL1 have been shown to negatively regulate Yap (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011a), the focus of this investigation was centered on understanding how Amot80 was regulating Yap alone and in combination with Amot130/L1. Cell lines with higher levels of detectable Amot130 seemed to display a greater sensitivity to Amot80 expression with respect to nuclear Yap activity. Cell lines with low or no detectable Amot130 showed significant but diminished responses to

Amot80 expression. All cell lines examined had relatively equal AmotL1 expression. Co-expression of Amot80 with Amot130 or AmotL1 consistently revealed a redistribution of Amot130 and AmotL1 to Amot80 induced internal puncta, suggesting a dominant role for Amot80 in regulating the location of Amot family members and Yap (Figure 4-9).

Total Yap levels did not reproducibly increase following Amot80 expression, however, the fraction of Yap phosphorylated on S127 did decrease every time Amot80 was expressed. Further, Amot silencing consistently reduced total Yap levels. These data suggest that Amot80 may either be dampening global Hippo signaling, physically separating Yap from Lats1/2 phosphorylation, or some combination of both. The Amot80 mutant lacking the PDZ binding domain was still able to redistribute Yap to Lats1-excluded endosomes, and increase nuclear Yap activity, however, the fraction of S127 phosphorylated Yap was not diminished compared to control. These data suggest that Amot80 expression dampens global Hippo signaling by disrupting apical polarity. Given that Amot80 WT and Amot80 $\Delta$ C both redistribute Yap, and increase its nuclear activity, the physical redistribution of Yap away from cell junctions is also effecting Yap transcriptional activity. These data are consistent with many reports which indicate Yap is regulated through several disparate mechanisms including Hippo signaling, actin cytoskeletal networks, and apical polarity complexes (Boggiano and Fehon, 2012; Spadaro et al., 2012).

Loss of Amot expression resulted in decreased levels of Yap protein regardless of cell type or method of Amot silencing. These findings suggest that Amot plays a positive role in maintaining Yap stability. Amot130 expression results in Yap redistribution to cell-cell contacts where Yap may be stabilized. Since Amot80 expression alone was insufficient to reproducibly increase Yap it is likely that Amot130 plays an important role in maintaining Yap levels. The protein levels of the functional homologue of Yap, transcriptional coactivator with PDZ binding motif (Taz) do increase following Amot silencing. These data are significant given that Yap and Taz both use TEAD to upregulate the transcription of CTGF and Cyr61 (Zhao et al., 2010a).

Although Yap and Taz proteins were initially considered redundant, their respective roles in promoting growth and de-differentiation have recently gained clarity (Cordenonsi et al., 2011; Lei et al., 2008).

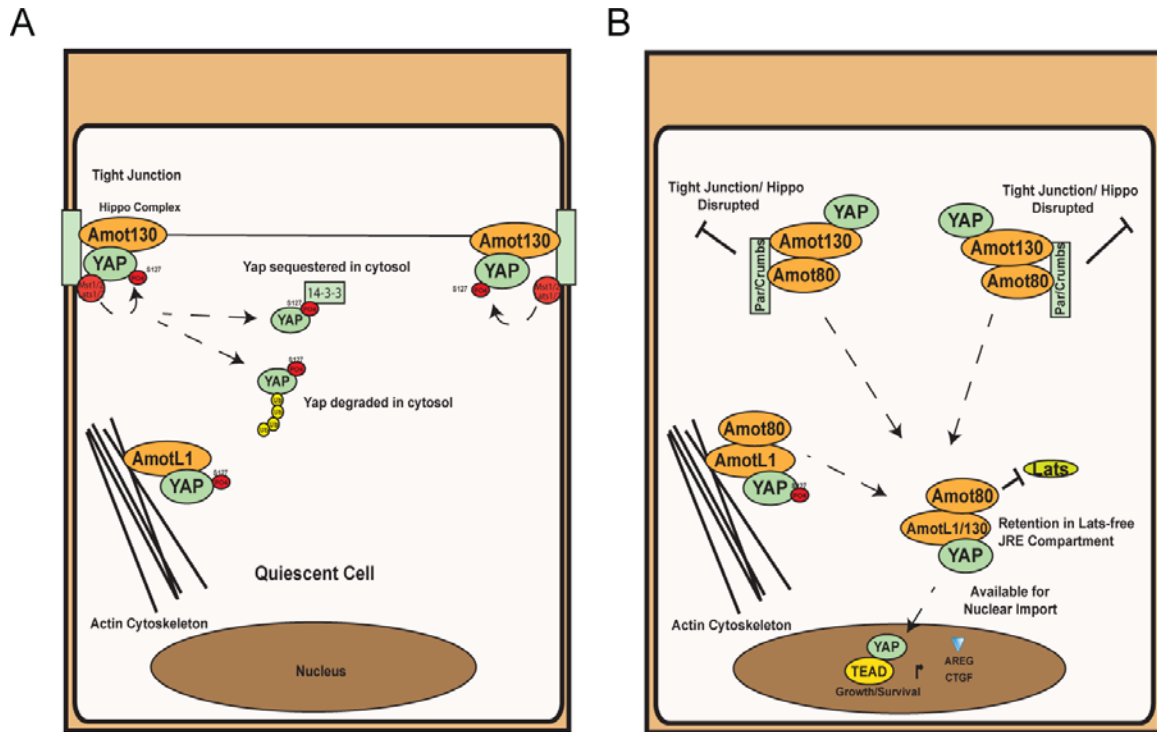
#### 4.3.3. Regulation of Amot Family Members During Acini Formation.

Elevated levels of Amot mRNA expression positively correlates with metastatic potential and tumor grade in mammary cancer (Jiang et al., 2006). The above results indicate that Amot80 expression promotes Yap transcriptional activity which is consistent with the role of Amot 80 in promoting growth (Ranahan et al., 2011). The immunofluorescence analysis from human mammary tissues suggests Amot distribution may be disrupted during tumor formation (Figure4-5B). Amot expression in normal ductal tissues is apical and confined to the luminal cells. Amot expression in tumor tissue appears to be aggregated within the cell and upregulated in cells peripheral to the duct. The presence of Amot in the basal layer of ductal tissue may be significant as this is the area thought to be the source of breast cancer tumor initiating cells (BTICs) following inactivation of the Hippo pathway (Cordenonsi et al., 2011) (Duss et al., 2012).

2D plastic substrata poorly recapitulate the microenvironmental cues regulating glandular tissues. The use of alternative culturing methods, such as Matrigel<sup>TM</sup>, provides an additional layer of physiologically relevant regulation (Kenny et al., 2007). In this context, AmotL1 expression is dynamically regulated during acini formation, whereas the same MCF10A cells grown on plastic maintain steady AmotL1 levels. Amot80 protein levels are not detectable in MCF10A cells grown on plastic but become apparent within 24 hours post transfer into Matrigel<sup>TM</sup>. These data indicate that the microenvironmental cues regulating gene expression are both contextually and temporally regulated.

Yap is the functional endpoint of several layers of regulation that coordinate cell-cell contact mediated inhibition of growth. Within the context of mammary cancer development, it is essential to understand how the function of Yap is regulated within the epithelial cells lining mammary ducts. Yap independently regulates organ size across many species (Dong et al.,

2007), and is aberrantly expressed in mammary tumors (Zhang et al., 2009). The epithelial cells lining mammary ducts are constantly responding to growth and apoptotic signaling and are the source of most mammary tumors. It is therefore critical to study a model system which incorporates the dynamic processes found *in vivo*. The best model, and most widely available, is MCF10A cells grown as acini in Matrigel<sup>TM</sup>. Unlike cells grown on plastic substrata, cells grown in Matrigel<sup>TM</sup> develop through synchronized growth and differentiation stages. Immunoblot analysis of these acini, during growth and differentiation, reveals a differential and timed expression of Amot80/130 and AmotL1 (Figure 4-5A). AmotL1 expression directly correlates with decreased levels of total Yap protein as well as increased Yap S127 phosphorylation. Loss of AmotL1 results in increased Yap transcriptional activity and acini overgrowth (Figure 4-7A). Amot80/130 expression remains constant throughout acini formation and is required for their normal growth. Loss of Amot80/130 expression decreases Yap nuclear activity (Figure 4-6). Taken together these data strongly suggest Amot family members play unique and essential roles in mammary epithelia during ductal formation.



**Figure 4-9 Model for Amot80 Induced Transcriptional Yap Activity.**

A. Quiescent State: Apical junctions are intact and facilitate negative Yap regulation via Hippo kinase activation. Cytosolic Yap is phosphorylated on S127 and sequestered by 14-3-3 or coupled to proteasomal degradation. Amot130 sequesters Yap to cell-cell contacts while AmotL1 sequesters Yap to cytoskeletal networks. Amot80 expression is low. B. Pro-growth State: Amot80 expression is upregulated. Amot80 binds and internalizes apical polarity components including Amot130 and components of the Par and Crb complexes. Internalized apical polarity complexes fail to maintain apical polarity and Hippo signaling is inhibited. Amot80 internalizes Yap into peri-nuclear recycling endosomes where it is segregated away from Lats and available for nuclear import. Yap enters the nucleus where it complexes with TEAD transcription factors and promotes transcription of genes involved in growth and de-differentiation.

## **CHAPTER 5. CONCLUDING REMARKS**

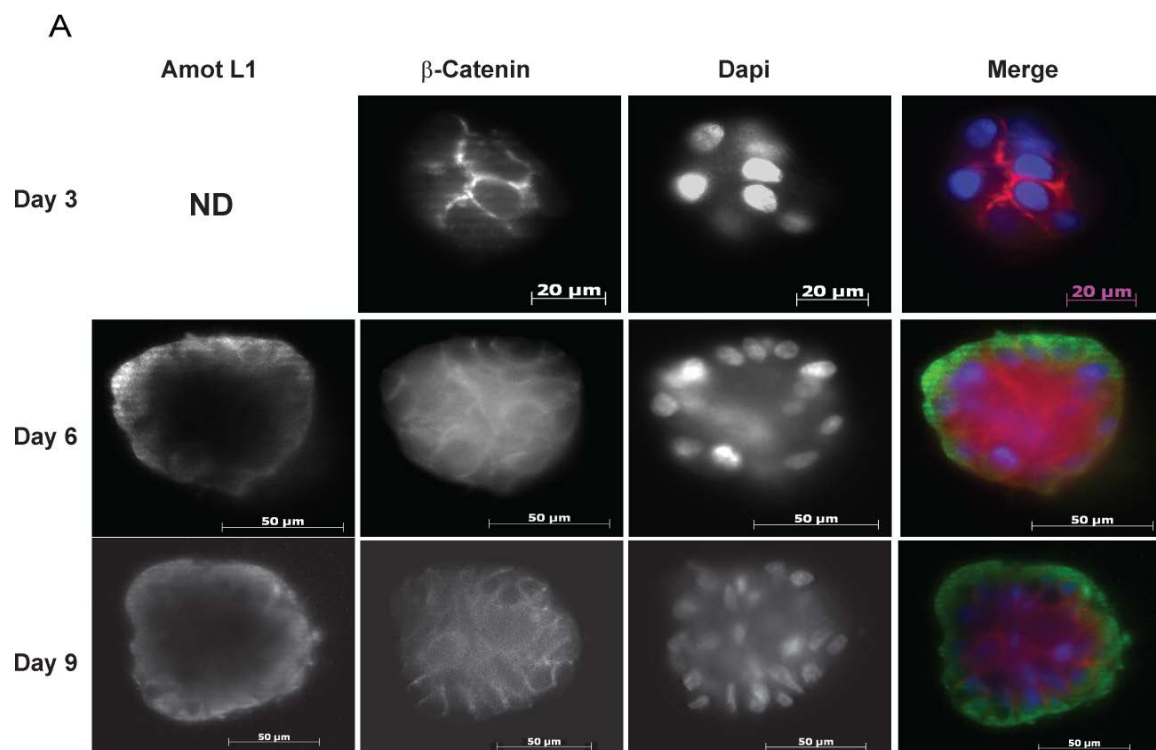
## 5.1. FUTURE STUDIES

### 5.1.1. Localization of Amot Family Members During Acini Formation

An important future direction will be to define cell type and subcellular location of Amots during acini development. Apical junctional integrity is regulated by the combined functions of junctional components, peripheral polarity complexes, and polarity adaptors (Assémat et al., 2008; Handler, 1989; Lene N. Nejsun, 2009; Shin et al., 2006). Correct localization of polarity complexes is essential for proper junctional maintenance. Amot redistributes polarity complexes thus regulates apical polarity (Heller et al., 2010; Ranahan et al., 2011; Wells et al., 2006).

Amot80 serves to disrupt the maintenance functions of Amot130 and Amotl1, but the maintenance functions of Amot130 and AmotL1 are poorly characterized. MCF10A cells grown in Matrigel as acini provide a polarized, growth arrested, physiologically relevant model for determining the locations of Amot130 and AmotL1. Location often gives clues as to function therefore understanding where a polarity adaptor is can shed light on its function. It is currently unclear what functions Amot130 and AmotL1 play during acini development. AmotL1 appears to be involved in suppressing growth at least partially through Yap inhibition; however, it is unlikely that Yap inhibition is the sole function of AmotL1. The role of Amot130 during acini development is completely opaque. One idea in the field is that Amot130 is growth inhibitory given its negative regulation of Yap but this can hardly be the sole function of Amot130. No study has yet focused on the coordinated regulation of growth by Amot family members. Technologies such as 3D matrix growth of acini and immunofluorescence microscopy are powerful tools which ought to be used in tandem. The mammary duct is not comprised of a monolayer of epithelial cells and therefore should not be solely investigated as such. Preliminary results suggest definite AmotL1 distribution patterns during acini development and provide a proof of principle that 3D imaging can produce meaningful data (Figure 5-1).





**Figure 5-1 Endogenous AmotL1 Expression During Acini Formation.**

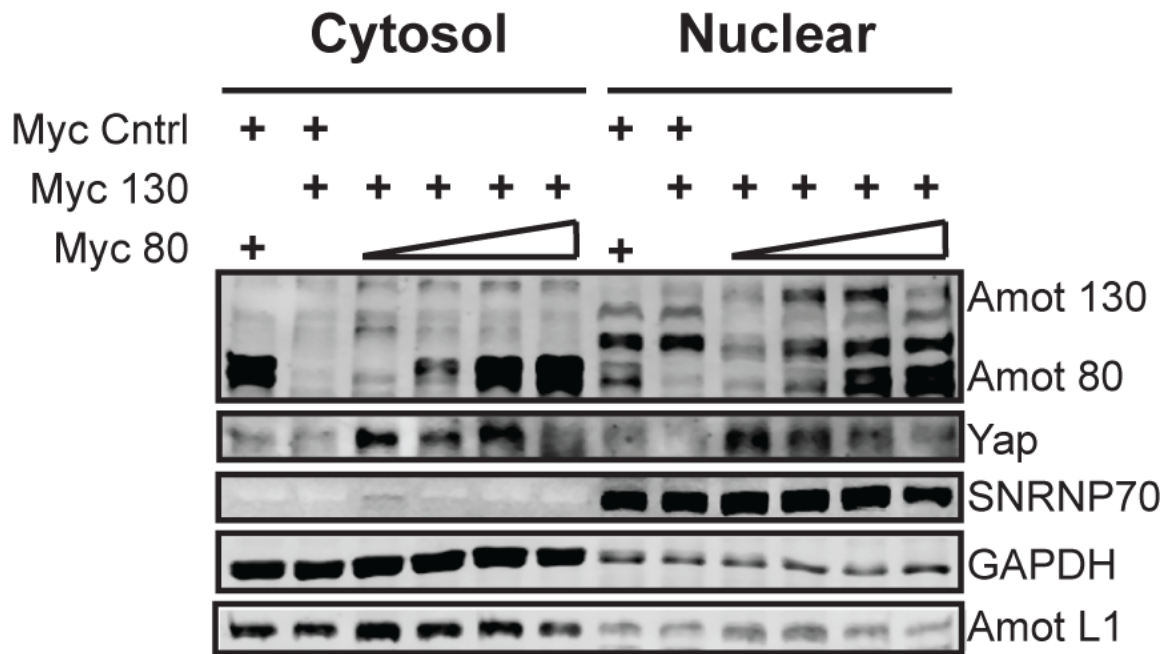
A. MCF10A cells were processed as previously described (Ranahan et al., 2011). Briefly, cells were seeded onto matrigel (BD Bioscience) coated collagen treated filters. Acini were MetOH fixed and subjected to immunofluorescence microscopy at indicated time points. Acini were immunolabelled with AmotL1 (green) and  $\beta$ -catenin (red). Nuclei were stained with Hoescht. ND indicates No Data.

### 5.1.2. Nuclear Localization of Amot Family Members

The proteins  $\beta$ -Catenin and ZO1 have established roles regulating apical junctional integrity and have been reported to translocate to the nucleus (Feigin and Muthuswamy, 2009; Huang and Muthuswamy, 2010; Royer and Lu, 2011). Preliminary data suggests that Amot family members may have similar nuclear translocation abilities (Figure 5-2). Amot80 expression localizes to endocytic recycling compartments where polarity proteins, phosphorylated Erk1/2, and Yap have all been identified (Ranahan et al., 2011) Figure S3E, (Figure 3-4) and (Figure 4-2) respectively. Amot80 expression enhances mammary epithelial cell growth by facilitating Erk1/2 and presumably Yap transcriptional activity.

The mechanism of Amot80 induced nuclear Erk1/2 and Yap translocation is unknown. One possibility is direct transport. Nuclear fractionation experiments reveal a propensity of endogenous Amot130 in the fraction enriched for nuclear extracts. AmotL1, on the other hand, appears to be mainly confined to the cytosolic fraction. Amot80 appears in both the nuclear and cytosolic fractions. A figure representative of several cell types is given below (Figure 5-2). These data suggest that much like ZO1, Amot130 can facilitate multiple cellular functions depending on physiological need. While cells are maintaining quiescence, Amot130 may function to promote Hippo signaling and proper localization of apical polarity complexes. During growth, however, Amot130 may be redirected into the nucleus, perhaps by engaging with Amot80. Any associated proteins, such as Yap, would then be included in this translocation to the nucleus. Thus Amot130 may adapt polarity proteins to maintain apical integrity while also being able to adapt transcriptional coactivators to their nuclear transcriptional complexes. This would be consistent with Amot130 expression resulting in sequestration of Yap at cell-cell contacts and loss of Amot130 resulting in growth defects and reduced Yap total protein levels.

A

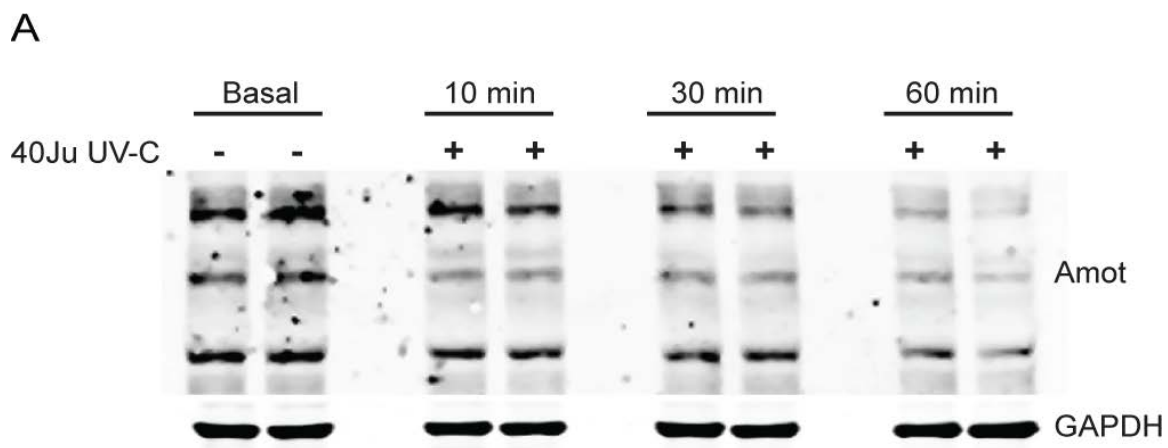


**Figure 5-2 Nuclear Localization of Amot80 and Amot130.**

A. MDAMB-468 cells were transiently transfected with control Myc or Myc tagged Amot80 and Amot130. Cells were subjected to nuclear fractionation before SDS-PAGE resolution of proteins and immunoblot analysis.

### 5.1.3. Post Translational Modifications of Amot

Amot is phosphorylated by ataxia telangiectasia mutated/ ataxia telangiectasia and Rad-3-related (ATM/ATR) in response to DNA damage induced by UV irradiation (Matsuoka et al., 2007). Amot is also ubiquitinated by the Nedd4 family of ubiquitin ligases (Adler, 2013 manuscript submitted). The physiological regulation of Amot will undoubtedly give insights into its functions within the cell. Consistent with Amot130 sequestering Yap to cell-cell contacts, Amot130 also mediates Yap ubiquitination by coupling Yap to Nedd4 (Adler, 2013 manuscript submitted). Yap has been shown to promote apoptosis under select cell stress conditions such as serum deprivation (Oka et al., 2008). Given that Amot130 binds Yap and can translocate to the nucleus, Amot130 may be involved in promoting apoptosis. Subsequent to UV induced DNA damage, Amot130 is phosphorylated in proximity to its PPXY motif (Matsuoka et al., 2007) where it associates with Yap (Zhao et al., 2011a). This phosphorylation event may disrupt Nedd4 association allowing a stable Amot130-Yap complex to be translocated to the nucleus, perhaps through Amot80. Preliminary data suggest that mammary epithelia respond to UV irradiation by down regulating Amot130 and to a lesser extent Amot80 (Figure 5-3). The mechanism of this loss of detectable Amot protein following UV insult is unclear and should be investigated further.



**Figure 5-3 Amot Levels are Effected by DNA Damage.**

A. MDAMB-468 cells were subjected to 40Ju UV-C or no treatment then harvested at indicated time points. Whole cell lysate proteins were resolved by SDS-PAGE followed by immunoblot analysis to measure the levels of Amot and GAPDH.

#### 5.1.4. Regulation of Amot Expression

Amot80 disrupts apical polarity by binding to and internalizing Par and Crb polarity complex members (Wells et al., 2006). Amot80 redistributes these polarity proteins to juxtanuclear recycling endosomes (Heller et al., 2010). These compartments facilitate prolonged Erk1/2 activation and promote growth (Ranahan et al., 2011). Amot80 expression also promotes Yap transcriptional activity, likely through redistribution of Yap from cell-cell contacts into these same intracellular compartments. Since Amot80 has such effects, it is important to understand the regulatory mechanisms of Amot expression. The Amot gene encodes two isoforms, the full length 130kDa and the N-terminally truncated 80kDa isoform. To date there have been no studies describing the mechanisms governing how one isoform is preferred over the other. In addition, no studies have been published revealing what stimulates Amot expression. Analysis of the promoter region of the Amot gene reveals predicted signal transducer and activator of transcription 3 (STAT3) binding sites suggesting Amot expression may be triggered by inflammatory cytokines such as TNF $\alpha$ . This is consistent with Amot transcripts being upregulated at the site of cutaneous wounding in mice (Roy et al., 2008). The putative induction of Amot by inflammation is particularly significant since the relationship between inflammation and breast cancer progression is gaining appreciation (DeNardo and Coussens, 2007). A causative role for Amot in promoting aberrant mammary epithelial growth is herein demonstrated. These data beg the question of how expression of Amot80 is upregulated under physiological conditions.

## 5.2. CONCLUSION

Breast cancers include a wide range of diseases of the breast, with each case presenting a unique challenge to treatment. Current treatments focus on targeting specific receptors expressed by cancer cells, e.g. Her2. Aberrant Her2 signaling often confers growth advantages to tumor cells through MAPK signaling, thus promoters of MAPK signaling should be considered for therapeutic targeting. The mammary organ is able to undergo pregnancy lactation cycles throughout the reproductive life of each woman. This unique sensitivity of the mammary organ to both growth and apoptotic signaling may underlie its proclivity to form tumors. The mammary gland that is not in the PLC is highly organized with epithelial polarity conferring a significant barrier to aberrant growth. During the PLC this organization, both intracellular and as a whole organ, must be reoriented to allow for de-differentiation of cuboidal epithelial cells into lactocytes, and expansion of the ductal networks. Tight junction integrity is especially amenable to change during the PLC which suggests that internal signaling paradigms exist which mediate tight junction stand dynamics. The tight junction is the apical most barrier to the diffusion of proteins and lipids and is required for the sequestration of growth receptors from their agonists. Tight junction integrity is mediated by polarity protein complexes which are in turn regulated by polarity adaptor proteins. Mammary epithelial cells must overcome contact mediated inhibition of growth in order to initiate tumor progression. Disruption of apical junctional integrity is directly linked to activation of loss of contact mediated inhibition. This dissertation presents data which demonstrates that expression of the adaptor protein Amot80 can disrupt tight junction integrity and lead to aberrant growth in mammary epithelial cells.

Current breast cancer treatments, such as ER+ tumors being treated with Tamoxifen, focus on targeting cell-surface receptors known to be associated with aberrant growth. However, a wide range of genetic and molecular profiles exists within the cells comprising ER+ tumors. Tamoxifen treatment reduces tumor mass but is not thought to eliminate all tumor cells. Cancer

stem cell theory suggests that the cells responsible for tumor generation are themselves genetically and molecularly distinct from the tumor cells they generate. A more universal marker is needed both for diagnosis and treatment.

Loss of intracellular polarity is one such marker. Cancer stem cells as well as invasive and non-invasive aberrantly growing epithelial cells, all present with diminished internal organization. Epithelial intracellular organization is regulated on three distinct levels. On the ground level, biophysical interactions between cells are mediated by transmembrane proteins forming homophilic extracellular interactions. Proper localization and maintenance of these transmembrane proteins, the middle level, is facilitated by polarity protein complexes. Polarity protein complexes are properly localized by the top level of regulation i.e. polarity protein adaptors. Amot is a polarity protein adaptor and thus functions at the top of the epithelial intracellular organizational pyramid. Changes in the ratio of Amot130 and Amot80 appear to determine whether polarity will be reinforced or lost.

Amot expression in breast cancer correlates with tumor grade and metastatic risk as well as poor patient outcome. This dissertation focuses on understanding the physiological results of Amot80 upregulation in mammary epithelia. Elucidation of the early events predisposing ductal epithelial cells to loss of polarity and growth has been the overarching goal. MCF10A cells grown in Matrigel<sup>TM</sup> recapitulate many of the signaling paradigms active during mammary duct development. Amot80 expression in this system results in acini overgrowth suggestive of a DCIS-like state. The most common type of non-invasive breast cancer is DCIS. DCIS is considered a precursor to IDC. Amot80 may therefore be a causal factor in the early events leading to breast cancer development. Breast cancer treatment efficacy dramatically increases when the disease is diagnosed early. Prognostic indicators of breast cancer are therefore highly relevant.

Several key questions remain to be answered, such as what upregulates Amot80 expression, and is Amot80 upregulated via genetic transcription or stabilized at the protein level?



The stoichiometry between Amot80 and Amot130 proteins has important consequences in endothelial and epithelial cells, but little is understood with respect to how this differential expression is controlled. The upregulation of Amot following cutaneous wounding suggests inflammation may trigger Amot expression but it remains unclear if this upregulation is specific to one family member or one Amot isoform. Given the oncogenic properties of Amot80 detailed above, the question of whether Amot80 expression could be used as an early indicator of breast cancer progression is highly significant.

On a final note, Yap and Taz have been implicated in the promotion of the cancer stem cell phenotype (Cordenonsi et al., 2011; Lian et al., 2010). Amot80 promotes Yap transcriptional activity. Expression of Amot increases in the basal layer of mammary ductal tumor tissues, that is, the area thought to originate cancer stem cells which drive DCIS (Cordenonsi et al., 2011) (Duss et al., 2012). Taken together these observations suggest Amot80 may be coordinating cell growth with de-differentiation in mammary epithelia. Amot80 appears to function as an oncogene in mammary epithelia. Amot80 may serve to promote growth and migration of epithelial tissues within the context of wound healing and it is likely that this normal physiological process is being hijacked during tumorigenesis.

## APPENDIX – NUCLEOTIDE SEQUENCES

### qRT-PCR PRIMERS

CTGF Forward: AGGAGTGGGTGTGTGACGA

CTGF Reverse: CCAGGCAGTTGGCTCTAATC

Cyr61 Forward: CCAGGCAGTTGGCTCTAATC

Cyr61 Reverse: CCAGGCAGTTGGCTCTAATC

GAPDH Forward: CCAGGCAGTTGGCTCTAATC

GAPDH Reverse: CCAGGCAGTTGGCTCTAATC

### siRNA SEQUENCES

Amot: AAGAAAAGCGAGACGACAAUU (Dharmacon Inc.)

Control ON-TARGET plus #1 D-001810-01-20, (Dharmacon).

### shRNA SEQUENCES

Amot shRNA#1: GACAGAAATCCAGCGCGTCTCG in pLKO.1 (Addgene)

Amot shRNA#2: GAGGAGAATGTGATGAGACAT in pLKO.1 (Sigma Aldrich)

Scramble: shControl pLKO.1 plasmid #1864 (Addgene)

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Zhao, H., Huang, M., Chen, Q., Wang, Q., and Pan, Y. (2012b). Comparative gene expression analysis in mouse models for identifying critical pathways in mammary gland development. *Breast Cancer Res Treat* 132, 969-977.

Zhi, X., Zhao, D., Zhou, Z., Liu, R., and Chen, C. (2012). YAP Promotes Breast Cell Proliferation and Survival Partially through Stabilizing the KLF5 Transcription Factor. *The American Journal of Pathology* 180, 2452-2461.

## **CURRICULUM VITAE**

**William P. Ranahan**

### **Education:**

**8/07 – 7/13                      Doctor of Philosophy in Biochemistry and Molecular Biology**  
**Indiana University**

**1/01 – 4/05                      Bachelor of Science in Biology**  
**Magna Cum Laude Oral Roberts University**

### **Work/Research Experience:**

**10/05 – 4/06                      Research Associate, ZymoGenetics, Seattle, WA**  
**Department of Molecular and Cell Based Discovery**  
**Supervisors: Betty Haldeman and Joe Kuijper, Scientists**

Responsibilities: Optimization and troubleshooting of a problematic RNA amplification protocol, and other RNA protocols including RNA preparation, quantization, and quality control via the Agilent Bioanalyzer. First strand cDNA synthesis and quality determination by PCR. Other PCR methods including high-throughput PCR in 96 well plate format, optimization of primer sensitivity and specificity, PCR-based gene cloning and repair, primer design using design software in conjunction with NCBI Entrez to identify gene sequences and transcript landmarks such as intron locations. *E. coli* handling techniques including transformations and glycerol stock preparation. DNA methods including restriction digests, ligations, mini, maxi and mega plasmid preps and robotic 96 well plate plasmid preps. Also responsible for upkeep of large DNA and RNA archives, including a FileMaker Pro database with details and location information for each sample.

**8/04 – 4/05**

**Senior Research Project, “Identification of Differentially Expressed Genes of Murine Inner Cell Mass and Embryonic Stem Cells.” ORU, Tulsa, OK**

**Advisor: Dr. Sarah Myer**

Responsibilities: Identified research issue, collected and reviewed current articles, prepared and defended research to senior biology class and biology faculty as well as to the BBB Honors Society Annual Regional Meeting. Techniques used include: PCR, cloning, plasmid mini-preps, gene identification via software and online search engine NCBI Blastn, familiarity with suppressive subtractive hybridization and real time PCR.

**1/04 – 4/05**

**Laboratory Assistant for Genetics, Molecular Cell Biology and Immunology courses, Department of Biology, ORU, Tulsa, OK**

Responsibilities: Coordinated and supervised the following - PCR, gel electrophoresis, western/southern/northern blots, column chromatography. Lab equipment maintenance and use; agar and protein mix preparations; live organism preparation (*Drosophila*, *E. coli*); solution preparations. Helped students understand and perform molecular techniques and lab safety protocol.

**8/04 – 4/05**

**President of Mu Kappa chapter of Tri-Beta National Biological Honor Society, ORU, Tulsa, OK**

Responsibilities: Organized weekly meetings to plan outreaches, research opportunities, guest speakers, and graduate research preparedness.

**2/04 – 4/04**

**Research Assistant at Oklahoma State University’s Center for Health Sciences, Tulsa, OK**

Responsibilities: Mini-prep purification of plasmid DNA, restriction enzyme digests, gel electrophoresis.

### **Awards:**

- 5/10 David M. Gibson Award 1<sup>st</sup> Place, Sigma Xi Research Competition
- 5/10 First Place Award, Basic Science Category, IU Simon Cancer Center, Annual Cancer Research Day
- 4/10 Outstanding Oral and Poster Presentation, 2010 Biochemistry Research Day
- 2/05 Who's Who Among American Colleges and Universities
- 10/04 Research grant from Tri-Beta, National Biological Honor Society

### **Publications:**

- 4/13 **Ranahan, W. P.**, Heller, B.L., Bringman, L.R. and Wells, C. D. (2013). "Amot80 promotes Yap nuclear activity in mammary epithelia." (Manuscript written)
- 4/13 Jacob J. Adler, Derrick E. Johnson, Brigitte L. Heller, Lauren R. Bringman, **Ranahan, W.P.**, Andy Hudmon, Clark D. Wells (2013) "Serum Deprivation Inhibits Yes-Associated Protein and Cell Growth via Phosphorylation of Amot by the Large Tumor Suppressor Kinase" Proceedings of the National Academy of Science
- 2/13 Adler, J.J., Heller, B.L., Bringman, L. R., **Ranahan, W.P.**, Cocklin, R.R., Goebel, M.G. Oh, M., Lim, H., Ingham, R.J., and Wells, C.D. (2013) "Amot130 adapts Atrophin-1 Interacting Protein 4 to inhibit Yes-associated Protein signaling and cell growth" Journal of Biological Chemistry DOI: 10.1074/jbc.M112.446534
- 1/11 **Ranahan, W. P.**, Z. Han, W. Smith-Kinnaman, S. C. Nabinger, B. Heller, B.-S. Herbert, R. Chan and C. D. Wells (2011). "The Adaptor Protein AMOT Promotes the Proliferation of Mammary Epithelial Cells via the Prolonged Activation of the Extracellular Signal-Regulated Kinases." Cancer Research **71**(6): 2203-2211.
- 4/08 Sanford, J. R., P. Coutinho, J. A. Hackett, X. Wang, **W. Ranahan** and J. F. Caceres (2008). "Identification of Nuclear and Cytoplasmic mRNA Targets for the Shuttling Protein SF2/ASF." PLoS ONE **3**(10): e3369.